SEROLOGICAL ASPECTS OF TUMOUR IMMUNOLOGY

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CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

PREFACE

INTRODUCTION PAGE

1. EMERGENCE OF TUMOUR IMMUNOLOGY 1

2. SURVEILLANCE OF NASCENT TUMOURS 4
   a) Virally-induced tumours 4
   b) Carcinogen-induced tumours 10

3. NATURE VERSUS NURTURE AS ETIOLOGICAL FACTORS IN HUMAN NEOPLASIA 17

4. ESCAPE FROM IMMUNOSURVEILLANCE 21
   a) Inadequate recognition of tumour antigens 23
   b) Immunoresistance 25
   c) Immunodeficiency of the host 25
   d) "Sneaking through" 26
   e) Abberation in the immune response 27

5. EFFECTOR MECHANISMS IN THE IMMUNE RESPONSE TO TUMOUR ANTIGENS 28
   a) T-cell response 28
   b) Non-T-cell response 29
   c) Macrophage response 32
   d) Antibody response 33

6. AUGMENTATION OF IMMUNE RESPONSE TO TUMOUR ANTIGENS 36
   a) Action of adjuvants 36
   b) Activation of helper T-cells 39

7. PURPOSE OF THE PROJECT 40
   a) Immunoglobulin classes and sub-classes and immune phenomena 42
   b) Antigen against which anti-tumour antibody is directed 49

PAGE

c) Mechanisms by which antibody may influence tumour growth 50

d) Plan of the project 53

MATERIALS AND METHODS

1. ANIMALS AND ANIMAL HANDLING 55

a) Mice 55

b) Rabbits 56

c) Goats 56

d) Guinea pigs 56

e) Bleeding of laboratory animals 56

2. PRODUCTION OF ANTISERA 57

a) Techniques for production of purified myeloma proteins 57

b) Injection of rabbits with purified myeloma proteins ... 59

c) Solid-phase immunoabsorption of antisera 60

d) Assessment of specificity of immunoabsorbed antisera .. 61

e) Comments on production of antisera 61

3. CELL LINES 62

a) Origin 62

b) In vivo propagation of tumours 63

c) In vitro propagation of tumour cell lines 63

d) In vitro propagation of embryo cell lines 64

e) In vitro propagation of normal kidney fibroblasts 64

f) Storage of cells under liquid nitrogen 65

g) Tumour transplantation and measurement 65

4. ASSAY TECHNIQUES 66

a) Antiglobulin assay 66

b) Assay for cytostatic antibody 71

c) Titration of anti-C.parvum antibody by latex agglutination 72

d) Estimation of immunoglobulin class and sub-class levels in mouse serum 72

e) K-cell cytotoxicity assay 73

f) Plaque-forming cell assay for antibody to sheep red blood cells 74

g) Assay for antibody response to alum-BSA 74

PAGE

5. USE OF ISOTOPES 75

6. PRESENTATION OF RESULTS 76

7. ADJUVANTS USED 76

RESULTS

1. DEVELOPMENT OF ANTIGLOBULIN ASSAY 78

a) Variation of uptake of mouse immunoglobulin with the age of the tumour target cells 78

b) Sensitivity of antiglobulin method A compared with method B 78

c) Optimum dilution of reagents in antiglobulin method B.. 79

d) Examination of CBA fibrosarcoma target cell loss during antiglobulin method B 79

e) Absorption of reagents to plastic reaction wells in method B 80

f) Optimum dilution of reagents in antiglobulin method D.. 81

2. USE OF ANTIGLOBULIN ASSAY TO DETECT IMMUNOGLOBULIN REACTING WITH SYNGENEIC TUMOUR CELLS AFTER C.PARVUM ADMINISTRATION .. 82

a) Effect of C.parvum treatment schedule on immunoglobulin binding to tumour cells in vitro 82

b) Cellular basis for the production of immunoglobulin binding to syngeneic tumour cells in vitro after C.parvum administration 84

c) Effect of C.parvum on immunoglobulin bound to tumour cells in vivo 87

d) Effect of different strains of C.parvum in normal and tumour bearing mice on immunoglobulin binding to syngeneic tumour cells in vitro 88

e) Effect of different adjuvants in normal and tumour bearing mice on immunoglobulin binding to syngeneic tumour cells in vitro 89

f) Characteristics of serum immunoglobulin binding to syngeneic fibrosarcoma cells in vitro 89

g) Specificity of serum immunoglobulin binding to syngeneic fibrosarcoma cells in vitro 90

3. USE OF ANTIGLOBULIN ASSAY TO DETECT ANTI-TUMOUR ANTIBODY ELICITED WITHOUT THE INTERVENTION OF C.PARVUM 94

a) Production of anti-tumour antibody by excision of tumour burden 94

b) Production of anti-tumour antibody by hyperimmunisation with syngeneic tumour cells 95

PAGE

4. ESTIMATION OF SERUM IMMUNOGLOBULIN CLASS AND SUB-CLASS LEVELS AFTER C.PARVUM ADMINISTRATION 96

a) Effect of C.parvum treatment schedule on serum immunoglobulin class and sub-class levels 97

b) Cellular basis for the changes in serum immunoglobulin class and sub-class levels after C.parvum administration 97

c) Effect of different strains of C.parvum in normal and tumour bearing mice on serum immunoglobulin class and sub-class levels 98

d) Effect of different adjuvants in normal and tumour bearing mice on serum immunoglobulin class and sub-class levels 98

5. DETECTION OF ANTIBODY CYTOSTATIC FOR CBA FIBROSARCOMA CELLS IN THE SERUM OF C.PARVUM-TREATED MICE 98

6. IMMUNOTHERAPY BASED ON THE PRECEEDING RESULTS 100

a) Active immunisation with embryo cells 100

b) Passive immunisation with serum from C.parvum-treated mice 101

7. ASPECTS OF THE IMMUNE RESPONSE IN MICE BEARING PLASMA CELL TUMOURS 102

a) Effect of syngeneic transplanted plasmacytomas on the antibody response of BALB/c mice 102

b) Effect of syngeneic transplanted plasmacytomas on the K-cell cytotoxic activity in the spleens of tumour bearing mice 103

DISCUSSION

1. CRITIQUE OF EXPERIMENTAL METHODS 105

2. SUMMARY OF RESULTS 108

3. RELEVANCE OF RESULTS TO TUMOUR IMMUNITY 115

ACKNOWLEDGEMENTS

PUBLICATIONS

REFERENCES

LIST OF TABLES AND FIGURES

TABLE		PAGE
1	Plasmacytomas used in these studies.	61a
2	Immunisation schedules for production of antisera to mouse immunoglobulin classes and sub-classes.	61b
3	Purification of IgA (one heavy + one light chain)	61c
4	Purification of IgM.	61d
5	Purification of IgG_1.	61e
6	Purification of IgG_{2a} (I).	61f
7	Purification of IgG_{2a} (II).	61g
8	Immunoabsorbent columns.	61k
9	Immunoabsorptions necessary before antisera were specific.	61l
10	Variations of antiglobulin assay (chronological order)	70a
11	Variation of uptake of mouse immunoglobulin with the age of CBA fibrosarcoma target cells.	81a
12	Comparison of antiglobulin methods A and B: variation of mouse serum dilutions.	81b
13	Optimum conditions for antiglobulin assay method B: variation of mouse serum concentration.	81c
14	Optimum conditions for antiglobulin assay method B: variation of rabbit anti-mouse IgG concentration.	81c
15	Optimum conditions for antiglobulin assay method B: variation of ^{125}I-globulin fraction of goat anti-rabbit IgG concentration.	81c

TABLE		PAGE
16	Estimation of cell loss during antiglobulin assay (Method B)	81d
17	Optimum conditions for antiglobulin method D: variation of concentration of antiglobulin reagent (^{125}I-labelled globulin fraction of goat anti-rabbit IgG)	81g
18	Optimum conditions for antiglobulin method D: variation of concentration of antiglobulin reagent (^{125}I-labelled globulin fraction of rabbit anti-goat IgG)	81h
19	The effect of the route of administration on the response to and anti-tumour properties of C.parvum in CBA mice	83b
20	The response of CBA mice to various doses of C.parvum injected i.p.	83e
21	The effect of gold salts on the response of CBA mice to C.parvum and its anti-tumour activity	86b
22	The effects of C.parvum in tumour bearing T-cell-deficient (B) mice	86d
23	Serological changes in "nude" mice after administration of C.parvum	86f
24	In vivo and in vitro tumour bound immunoglobulin in C.parvum-treated tumour bearing mice	88a
25	Uptake onto CBA fibrosarcoma cells of G-200 sephadex fractions of syngeneic serum, using antiglobulin methods B and D	93a
26	Uptake onto CBA fibrosarcoma cells of IgA and IgM from the serum of C.parvum-treated CBA mice	93b
27	Uptake onto CBA fibrosarcoma cells of IgG sub-classes from the serum of C.parvum-treated CBA mice	93c
28	Effect of preincubation with aggregated human IgG on the uptake of immunoglobulin onto tumour cells	93d

TABLE		PAGE
29	Specificity of antibody evoked by C.parvum (I)	93g
30	Specificity of antibody evoked by C.parvum (II)	93h
31	Specificity of antibody evoked by C.parvum (III)	93i
32	Summary of specificity data using normal and malignant target cells	93j
33	The effect of absorption with C.parvum, tumour and spleen on the antibody activity of mouse serum pools	93k
34	Appearance of anti-tumour antibody in CBA mice after immunisation with syngeneic tumour cells	95c
35	A comparison of the effects of C.parvum and P.freudenreichii on immunoglobulin levels and tumour growth	97d
36	A comparison of the effects of C.parvum and other adjuvants on immunoglobulin levels and tumour growth	97e
37	A comparison of CBA fibrosarcoma cells and syngeneic embryo cells as immunising agents against the CBA fibrosarcoma	101a
38	The effect of pretreatment with CBA mouse serum on the growth of transplanted syngeneic tumour cells	101c
39	Influence of transplanted plasmacytomas on the response to alum-BSA	104a
40	K-cell activity in mice bearing MOPC47A plasmacytoma	104c
41	K-cell activity in mice bearing MOPC104E plasmacytoma	104d
42	K-cell activity in mice bearing MPC25 plasmacytoma	104e

TABLE		PAGE
43	K-cell activity in mice bearing ADJPC5 plasmacytoma	104f
44	The effects of C.parvum in normal CBA mice: a summary	124a
45	The effects of C.parvum in tumour bearing CBA mice: a summary	124b
46	Possible sequelae to C.parvum injected systemically	124c

FIGURE		
1	The circular argument of immune surveillance	2a
2	Production of IgM	61h
3 - 4	Production of Fc fragment of IgG_{2a}	61i-j
5	Effectiveness of immunoabsorbent columns (1)	61m
6	Effectiveness of immunoabsorbent columns (2)	61n
7	Examination of specificity of rabbit anti-mouse (RAM) IgA	61o
8	Examination of specificity of rabbit anti-mouse (RAM) IgM	61p
9	Examination of specificity of rabbit anti-mouse (RAM) IgG_1	61q
10	Examination of specificity of rabbit anti-mouse (RAM) IgG_{2a} (I)	61r
11	Examination of specificity of rabbit anti-mouse (RAM) IgG_{2a} (II)	61s

FIGURE		PAGE
12	Examination of specificity of rabbit antisera to mouse immunoglobulins using commercial standards	61t
13	Specificity of rabbit anti-mouse IgG used in antiglobulin assay	70b
14	Calibration curve for estimation of IgG_{2a} in mouse serum	72a
15	Uptake of immunoglobulin by a) CBA tumour cells plus plastic reaction wells, b) plastic reaction wells only	81e
16	Uptake of immunoglobulin by CBA tumour cells (ie cells removed from the reaction vessel for counting)	81f
17	Effect of route of C.parvum injection on uptake of immunoglobulin by CBA fibrosarcoma cells in normal and tumour bearing mice	83a
18	Effect of different doses of C.parvum in CBA mice on the uptake of immunoglobulin onto CBA fibrosarcoma cells	83c
19	Immunoglobulin uptake from the serum of C.parvum-treated CBA mice onto CBA and A/HeJ fibrosarcoma cells	83d
20	Effect of gold salts on the uptake of immunoglobulin by syngeneic tumour cells by C.parvum-treated tumour bearing CBA mice	86a
21	Correlation between immunoglobulin taken up by CBA fibrosarcoma cells and anti-C.parvum antibody in thymus-deprived CBA mice	86c
22	Effect of C.parvum in congenitally athymic mice on serum immunoglobulin binding to CBA tumour cells	86e
23	Detection of immunoglobulin on the tumour cell surface and in autochthonous serum	88b

FIGURE		PAGE
24	Correlation between immunoglobulin detected on the tumour cell surface and in autochthonous serum	88c
25	Effect of different strains of C.parvum in normal and tumour bearing CBA mice on uptake of immunoglobulin onto syngeneic fibrosarcoma cells	88d
26	Specificity of anti-tumour antibody produced by C.parvum 10387 in CBA mice bearing a syngeneic fibrosarcoma	88e
27	Effect of different adjuvants in normal and tumour bearing CBA mice on uptake of immunoglobulin onto CBA fibrosarcoma cells	88f
28	Uptake of immunoglobulin from the serum of C.parvum -treated CBA mice onto syngeneic fibrosarcoma cells and embryonic fibroblasts	93e
29	Uptake of immunoglobulin from the serum of C.parvum -treated CBA mice onto syngeneic fibrosarcoma cells and spleen cells	93f
30	Appearance of immunoglobulin binding to CBA fibrosarcoma cells after tumour amputation (Expts. 1 and 2)	95a
31	Appearance of immunoglobulin binding to CBA fibrosarcoma cells after tumour amputation on Day 0 (Expt. 3)	95b
32	Uptake of immunoglobulin from the serum of mice immune to a syngeneic CBA fibrosarcoma (using antiglobulin method B)	95d
33	Uptake of immunoglobulin from the serum of mice immune to a syngeneic CBA fibrosarcoma (using antiglobulin method C)	95e
34	Effect of route of C.parvum injection on serum immunoglobulin levels in normal and tumour bearing CBA mice	97a

FIGURE		PAGE
35	Effect of different doses of C.parvum on immunoglobulin levels in CBA mice	97b
36	Effect of gold salts on immunoglobulin levels in C.parvum-treated tumour bearing CBA mice	97c
37	Cytostatic activity of serum from normal and C.parvum-treated CBA mice against syngeneic fibrosarcoma cells	99a
38	Cytostatic activity against CBA fibrosarcoma cells of serum from a) mice bearing a syngeneic fibrosarcoma, b) mice bearing the fibrosarcoma treated with C.parvum	99b
39	Dependence of the cytostatic effect of serum from C.parvum-treated tumour bearing CBA mice on extraneous complement	99c
40	Effect of immunisation with embryo cells on the growth of a syngeneic fibrosarcoma in CBA mice	101b
41	Influence of plasmacytoma growth on the response to SRBC	104b

SUMMARY

It has been postulated that antibody may affect tumour growth directly, or indirectly by modulating the activities of T-cells, K-cells and macrophages. Therefore, these studies were designed to gain some insight into whether the pleomorphism exhibited by immunoglobulin classes and sub-classes might account for the numerous roles that have been postulated for antibody in tumour immunity. The model chosen was the inhibition of syngeneic tumour growth (s.c.) by an injection of C.parvum (i.p.) in CBA mice, and three serological parameters were measured after administration of C.parvum, tumour or both. These were: 1) immunoglobulin binding to tumour target cells in vitro (sometimes designated "anti-tumour antibody", in an operational sense); 2) total immunoglobulin class and sub-class levels; 3) anti-C.parvum antibody titres.

With the aid of serum from appropriately immunised mice, an isotopic antiglobulin assay was developed to detect immunoglobulin binding to target cells in vitro. It soon became apparent that C.parvum (CN 6134) administration (i.p.) to normal mice resulted in the production of immunoglobulin binding to tumour cells in vitro, accompanied by elevated levels of certain serum immunoglobulins (most markedly and consistently IgG_{2b}) and high anti-C.parvum antibody titres. These serological changes were dose and route dependent, although not dependent on an intact thymus, and they occurred with other adjuvants. The immmunoglobulin binding to tumour cells in vitro was IgM, and exhibited no specificity for tumour cells.

C.parvum (CN 6134) administration (i.p.) to tumour bearing mice also elicited immunoglobulin capable of binding to tumour cells in vitro, accompanied by elevated levels of most immunoglobulin classes

and sub-classes and high anti-*C.parvum* antibody titres. Again the changes were route dependent and could be diminished (or, in the case of immunoglobulin binding to tumour cells *in vitro*, abolished) by the administration of gold salts. They were also apparent in thymectomised mice and in mice treated with other adjuvants. The immunoglobulin binding to tumour cells *in vitro* occurred in all classes and sub-classes except IgA, and the 7S immunoglobulin exhibited a degree of specificity for homologous tumour cells, although the 19S did not.

Preliminary experiments were also undertaken to see if; 1) the antibody detected by the antiglobulin assay was due to genuine antigen-antibody reaction; 2) immunoglobulin binding to tumour cells *in vitro* could be elicited without the intervention of adjuvant; 3) the serological changes could influence tumour growth; 4) tumours of lymphoid origin could affect the immune response to defined antigens.

The results are related to findings from other laboratories, and discussed from the standpoint of possible mechanisms of adjuvant action. Suggestions are made for improvement of techniques used and for further work in this area.

Systems, scientific and philosophic, come and go. Each method of limited understanding is at length exhausted. In its prime each system is a triumphant success: in its decay it is an obstructive nuisance. The transitions to new fruitfulness of understanding are achieved by recurrence to the utmost depths of intuition for the refreshment of imagination. In the end - though there is no end - what is being achieved, is width of view, issuing in greater opportunities.

from "Adventures of Ideas"
by Alfred North Whitehead

PREFACE

The discipline of tumour immunology is in a state of flux at present. Therefore to give this work some structure the hypothesis is adopted that immunosurveillance of sub-clinical neoplasms is a reality only for certain virally induced tumours, and is weak or non-existant for "spontaneous" and chemically induced tumours; susceptibility and resistance to an oncogenic agent being governed by non-immunological genetic factors in these cases. This does not preclude the possibility that tumours may be recognised by the host when the antigenic load is big enough, i.e. when the tumour is clinically observable.

INTRODUCTION

1. EMERGENCE OF TUMOUR IMMUNOLOGY

It has long been the experience of alert clinicians that the growth of an observable tumour is not always inexorable. Apart from the well attested cases of hormone-dependent neoplasms (1) there are a number of cases in which other, more capricious, mechanisms of homeostasis might be responsible. As long ago as 1891 W.B. Coley observed a connection between a stimulated immune system and regression of sarcomatous growth. Strangely, in spite of the celebrated work of his contemporaries Pasteur and Koch, he attributed the anti-tumour activity to a direct effect of the immune stimulant (S. pyogenes) on the cancer cells or "cancer bacillus" (2).

From then up to the present day there has appeared just enough evidence to keep the immunology of human tumours a live issue (3). This evidence usually takes the form of increased survival times, or duration of remission after immunotherapy. These are somewhat marginal for solid tumours but more dramatic for cancers in which the disease can be objectively controlled before commencement of immunotherapy, such as the various forms of leukemia. There are also well attested individual cases in which immunodepression has been followed by malignant outgrowth (4), or the association of a good prognosis with a high degree of lymphocyte infiltration into the regional node (5).

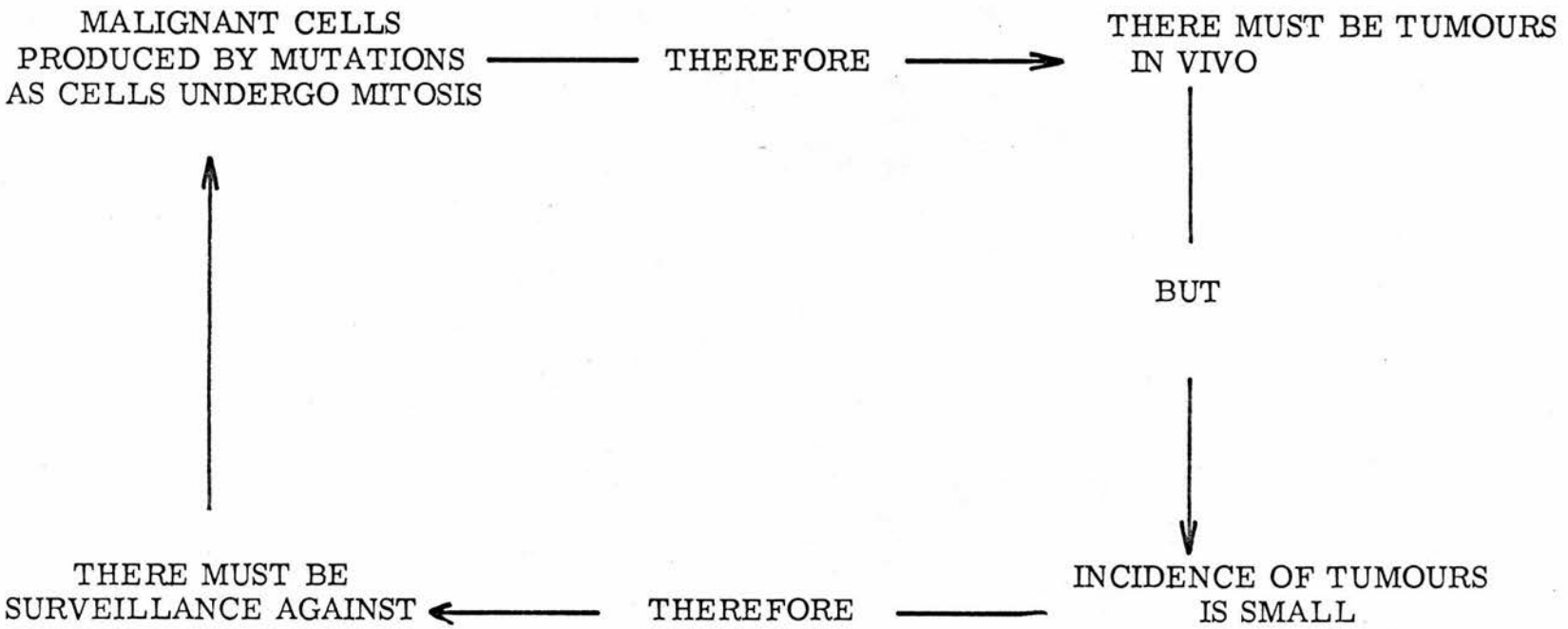
Tumour immunology acquired a theoretical basis in the immunosurveillance concept, due initially to Thomas (6) and subsequently to

Burnet (7). This concept, that the role of the thymus-dependent arm of the immune system was to seek out and destroy clinically unrecognised, *in situ* tumours which arose through somatic mutation, gave a satisfying explanation of the selective preasure culminating in the allograft reaction. It also accounted for the observations of increased tumour incidence associated with immune-depression or immune-disorder. With a few notable exceptions the theory was accepted and widely invoked in an explanatory capacity. It also made empirically testable predictions which have made it possible to adjudicate on the validity of the theory.

The results of the numerous experiments designed to test the immunosurveillance concept have recently been reviewed extensively, (see for example 8) and it seems apparent the experimental prediction, that immunosuppression should lead to a greater yield of all types of tumour, has been fulfilled only in a very restricted sense. Thus there is evidence that surveillance operates against widespread, potentially oncogenic viruses in a number of species, for example polyoma virus in mice, herpes virus saimiri in squirrel monkeys, Marek's disease virus in chickens, feline leukemia virus in cats and Epstein-Barr virus in man. As regards carcinogen-induced tumours the evidence is contradictory, but on balance the effect of a carcinogen appears to be genetically defined at the level of the cell (or organ) exposed to the carcinogen; immune intervention making a marginal difference (9).

With the benefit of hindsight it is possible to see that the immunosurveillance concept is based on a circular argument (see Fig. 1); however, this would have been immaterial if the theory had

Fig. 1 THE CIRCULAR ARGUMENT OF IMMUNE SURVEILLANCE



been sound. But because it is the business of science to progress from hypotheses that have exceptions to hypotheses that have no exceptions, the surveillance concept must be reformulated to take account of the good correlation in some viral systems between an intact immune system and low tumour incidence, and the meagre effect of immune-intervention on the incidence of carcinogen-induced tumours.

Such a theory has been proposed by Klein (10, 11), the central tenet of which is that genetically determined variation in immune responsiveness to a given tumour-associated antigen may account for the difference between susceptibility and resistance to the oncogenic effect of a given agent. The effects of the immune response genes can range between states of complete unresponsiveness and high reactivity; they may influence the timing or the quantitative level of the response; or they may modify the effector mechanisms involved in the reaction.

The theory would predict that a species harbouring a potentially oncogenic virus would be subject to selective pressures, resulting in a high degree of immune responsiveness against cells transformed by the virus. Members of a similar species not harbouring the virus would be susceptible to the oncogenic effect of the virus. As discussed later this has been found to be the case.

What of tumours produced by carcinogen? These oncogenic agents are very recent additions to the mammalian environment and no selection of IR genes against carcinogen-induced tumours would be expected. Thus, the empirical evidence that the induction of carcinogen-induced tumours is only marginally influenced by immune

factors, is in accord with the theory of genetic preselection of IR genes. There is, however, no doubt that under certain experimental conditions (e.g. large doses of carcinogen, manipulation of tumour presentation to the host) and on certain genetic backgrounds, there is excellent recognition by the host of carcinogen-induced tumour antigens; on the other hand sometimes there is no recognition at all. This random, and comparatively weak, recognition could be explained by assuming that the well recognised carcinogen-induced tumour antigens are those that cross-react with some natural product for which the host possesses the relevant IR genes.

As a footnote, it might be added that the genetic preselection theory does not provide an explanation of the selective pressures culminating in the allograft reaction, as did the immunosurveillance concept of Burnet. However, this in itself is not necessarily against the preselection theory as other explanations of the basis of the allograft reaction have been put forward (12).

2. SURVEILLANCE OF NASCENT TUMOURS

a) Virally induced tumours

As stated in the preceeding section, there is evidence for surveillance of sub-clinical, virally induced neoplasms in certain well defined systems. In some of these systems it has been possible to breed animals that are susceptible to the oncogenic effect of the virus (or virus-transformed cells), and animals that are resistant, thereby permitting a genetic analysis. When this has been done it has generally been found that histocompatibility linked genes are associated with susceptibility or resistance.

(This may implicate IR genes, but it is realised that histocompatibility genes may well be linked (or be identical) to genes coding for receptors on the cell surface, thereby perhaps influencing the severity of virus infection.) Furthermore, in some systems the difference between resistance and susceptibility can be abrogated by immune intervention.

i) Surveillance of virally induced tumours in mice.

Possibly the most studied system in tumour immunology is the induction of tumours with polyoma virus. As the virus is a ubiquitous constituent of wild mice this model may be particularly relevant. There is agreement that immunodepression of inbred laboratory mice (treatment with anti-lymphocyte serum or by thymectomy) results in an increased tumour yield after infection with polyoma virus, and this can be at least partially reversed by reconstruction of the immune system (9, 10). Furthermore, "nude" mice and "immunodepressed" neonates are similarly susceptible, all of which is strong evidence for the classical surveillance concept. The fact that wild type neonates do not show a high incidence of polyoma-induced tumours could be due to the prophylactic effect of maternally transferred antibody to polyoma virus, a protection not available to neonates of polyoma-free laboratory mice. The polyoma system appears to be reasonably typical of other oncogenic DNA viruses such as adeno-virus-12 and SV-40.

There are also laboratory analogies for some of the "natural" C-type RNA viruses of wild mice. Thus, in the Gross virus system there is some evidence that the difference between susceptible and resistant strains is due to a gene located in the

IR region of the H-2 complex. Most mouse strains carry the resistance allele, but the susceptibility allele can be selected for, e.g. in AKR mice (10). In the Friend virus system two genes have been defined. One determines recovery from leukemia and is located in the H-2 region; the second determines recovery from viremia and is due to three or more $C_{57}Bl$ genes independent of the H-2 complex. These $C_{57}Bl$ genes were necessary for the expression of the H-2 mediated effect on regression (13). It is interesting that although $C_{57}Bl$ mice differing at the H-2 linked gene both recover from viremia, the rate of recovery is faster in the mice with the gene coding for recovery from leukemia (13). This may indicate that the timing of recovery from viremia is critical. If it is too late the neoplastic transformation may be too far advanced to halt.

In general it can be said that immunosuppression increases the yield of tumours induced by the C-type viruses. However there are complications; for example thymectomy may remove the target cell on which the virus exerts its oncogenic effect, and ALS may react with the transformed cells.

ii) Surveillance of virally induced tumours in cats

Cats also harbour a C-type oncorna virus, feline leukemia virus (FeLV). When a cat is infected the virus is first located in the bone marrow, and subsequently in the mucous membranes of the respiratory and alimentary tracts. From there the virus particles are shed, and infection of other cats by horizontal transmission occurs (14). An important

consequence of FeLV infection is the onset of a state of immunosuppression so that the cats receiving a heavy infecting dose do not mount a substantial immune response, and may subsequently develop tumours. Conversely, cats infected with a small dose of virus produce a good antibody response and are tumour free (15).

In Glasgow infection is rare below 5 months of age, but later, when cats mix socially, there is a rise in the infection rate, so that of animals over 3 years 70% have antibodies (16). Thus, thesituation of horizontal transmission and a high infection rate with relatively low morbitity would indicate that immunosurveillance is operative. However, the antigen recognised, and used in vaccination in this system, would appear to be a viral envelope antigen (15). This is different from say a Moloney virus-induced tumour, in which the tumour antigen and the viral proteins do not cross-react (17).

iii) Surveillance of herpes virus-induced tumours

A number of widely divergent animal species are thought to be susceptible to the oncogenic effect of herpes viruses: these are frogs, chickens, monkeys and man.

The leopard frog (Rana pipiens) is particularly susceptible to a kidney carcinoma, implying some genetic predisposition. The nucleii of some tumour cells contain inclusions which are thought to be caused by virus. Subsequently it was found that the inclusions were only observed at low temperatures, when the tumour cells released infectious virus. In warm temperatures, spontaneous tumours occur which do not release infectious virus.

The cycle can be completed by recovering the infectious agent from a tumour induced by injection of a tumour extract, and using this in turn to induce another tumour. Thus, Kochs' postulates are satisfied, and the tumour is placed among those transmitted by an infectious agent (18).

In chickens, cells of the lymphoid system may become cancerous and invade the nervous system, resulting in paralysis. The disease, Marek's disease, progresses to other organs and the bird eventually dies. Tumour cells apparently do not contain virus but cells of the feather follicles do. Furthermore, it can be shown that chicken feathers and bits of sloughed off skin are highly infectious, thereby establishing the route of transmission (19). It is possible to breed chickens resistant and susceptible to the oncogenic effect of the virus, and neonatal thymectomy converts resistant birds to susceptible. Furthermore, resistance may be coded for by a single dominant gene which could well be an IR gene that influences recognition of the virus-transformed cells (10).

Vaccination of susceptible chickens with an apathogenic turkey herpes virus dramatically reduces the tumour incidence, without affecting the degree of virus shedding (20). This neatly illustrates two points: firstly, in Marek's disease it is impossible to immunise with the oncogenic virus (compare the polyoma system), presumably because the recognition of virally induced tumour antigens is not as good as in the polyoma system. However, if the timing is right, susceptible birds can be alerted to mount a tumour rejection response. Thus, susceptible birds can

respond, but their primary response is overwhelmed by the developing tumour. Secondly, the rejection response must be due to recognition of tumour antigens and not anti-viral immunity.

In monkeys we have the nicely defined situation in which hepes virus saimari, which exists in an uneventful manner with its natural host, causes a malignant lympho-proliferation in other species of monkey. Primary virus infection leads to comparable peak anti-virus antibody titres; however, the natural host responds more promptly than the susceptible species (21). This is similar to the Friend leukemia virus infection of susceptible and resistant strains of mice discussed above, and is evidence for the genetic control of resistance to the oncogenic effect of viruses. That this control takes place by an immune reaction against the transformed cell, and not by anti-viral immunity, is indicated by the fact that continuous lymphoblastoid cell lines containing the viral genome can be isolated from both resistant and susceptible species of monkey infected with virus.

In man Kochs' postulates cannot be fulfilled, so the implication of viruses in human tumours is essentially guilt by association and extrapolation from animal models. There are, however, a number of observations which indicate that the relation of Homo sapiens and the Epstein-Barr virus (EBV) is akin to some of the surveillance models previously discussed:-

i) 80 - 90% of adults harbour the virus, in all geographical locations (22), and consequently have high anti-viral antibody titres.

ii) Human B-lymphocytes can be "immortalised" in tissue

culture after infection with EBV. Such cells carry the EBV genome and grow progressively in immunosuppressed animals (23).

iii) In young adults there occurs a self-limiting, lymphoproliferative disease, infectious mononucleosis, that is caused by a virus indistinguishable from EBV. Comparing sero-positive subjects (high anti-viral antibody levels) with sero-negative, it was found that no infectious mononucleosis cases presented in the sero-positive group, but in the sero-negative group 10% developed the disease accompanied by sero-conversion (24).

iv) The EBV viral genome was found to be associated with Burkitt's lymphoma. Burkitt like or non-Burkitt like lymphomas outside Africa contained no detectable viral genomes, including lymphomas from patients who harboured the virus (10).

v) EBV carrying cell lines can be established from the blood of healthy sero-positive individuals (25).

Thus, it is tempting to suppose that a surveillance mechanism is policing the EBV-transformed cells. The mechanism may malfunction partially in infectious mononucleosis, or completely in Burkitt's lymphoma.

b) <u>Carcinogen-induced tumours</u>

For carcinogen-induced tumours surveillance cannot be demonstrated as convincingly as for virally induced tumours (for review see 9). It has been suggested that this may be due to the immunodepressive effect of the carcinogens themselves, which makes further immunodepression ineffective in increasing tumour incidence. This suggestion can be disposed of as the immunodepressive and oncogenic

effects of chemical carcinogens can be readily dissociated on the basis of dosage; both the immunodepressive and oncogenic effect being apparent at high doses, and only the oncogenic effect at low (9).

There is considerable evidence, from *in vitro* cytotoxicity tests and *in vivo* concomitant immunity, for an immune response against an established tumour (*vide infra*), so the problem may well turn on the question of how many cells constitute a nascent tumour as postulated in the original surveillance model. That is to say, there may be a threshold number of cells at which point immune recognition takes place, but by this time the tumour has a "head start". The alerted immune response may have a minor anti-tumour effect at this stage, but in the main will be irrelevant, or possibly tumour enhancing (26).

It has been pointed out that experimental evidence for surveillance of carcinogen-induced tumours is contradictory (9), making a synthesis impossible. Therefore, two seemingly unimpeachable experiments will be discussed which lend support to the notion that the effect of a carcinogen on an individual is decided by the genotype, at the level of the cell (or organ) which is subject to the insult of the carcinogen. However, there would appear to be a minor effect due to immunological intervention.

In a well controlled experiment Stutman (27) examined the induction of tumours by sub-immunosuppressive doses of methylcholanthrene in susceptible and resistant mice. After being tolerised to rabbit IgG both strains of mice were treated with rabbit anti-mouse ALG, and the incidence and latent period of tumours on application of methylcholanthrene was measured. It was observed that in susceptible mice the latent period and tumour incidence were the same in untreated

mice and mice treated with ALG or normal rabbit IgG. However, in the resistant strain ALG resulted in a doubling of tumour incidence and a slightly reduced latent period. Resistant mice treated with normal IgG, and untreated mice, had the same tumour incidence and latent period. This doubling of tumour incidence (from 1h% to 33%) after ALG treatment would indicate a role for surveillance in this system. However the major part of the resistance (67%) would appear to be due to non-immunological genetic factors.

In another system Heston et al. (28) transplanted lungs from strains of mice resistant and susceptible to carcinogen-induced lung tumour formation to the F_1 hybrid, and examined the incidence of tumour in the transplanted lungs. The susceptible strain and the resistant strain differed by at least four gene pairs and susceptibility was dominant. He observed that susceptibility and resistance were retained in the lungs when transplanted to the F_1 host, indicating that these genes controlled the degree of susceptibility through mechanisms limited to the lungs rather than some general systemic action. However, it may be worth noting that the incidence of tumour in susceptible lungs in susceptible hosts was slightly greater than with susceptible lungs in F_1 hybrids, indicating a minor influence on susceptibility by the F_1 host.

However, is tumour immunology dependent on the concept of immune surveillance of nascent tumours for its validity? Granted that surveillance against carcinogen-induced, clinically unrecognised, in situ tumours is weak or non-existant, does this mean that when the antigenic load is increased in a frank neoplasm, or the antigen is presented to the host in a suitable way, the host is still unable to

recognise it? This latter point was recently examined by Andrews (29) who showed that incipient tumours produced by methylcholanthrene were non-immunogenic unless the presentation of the tumour antigens to the host was altered by full thickness autografting of the area containing the methylcholanthrene pellet. Unfortunately this procedure was ineffective with non-antigenic tumours. Furthermore, the numerous studies of *in vitro* cell-mediated cytotoxicity (for review see 30) and the reports of concomitant tumour immunity (31) indicate that although surveillance of nascent carcinogen-induced tumours is ineffective, there may yet be antigens that can be exploited.

It is of considerable interest to note that both responsiveness to carcinogen-induced tumours, and indeed the tumour antigen produced by the carcinogen, may be controlled by the H-2 complex. Thus, the well known phenomenon of hybrid resistance or allogeneic inhibition can be explained by proposing better recognition of tumour antigens by certain genetic backgrounds containing the relevant IR genes. This would be analogous to the virally induced tumour systems discussed earlier, although the degree of recognition is much smaller. Williams *et al.* (32) have examined the growth of a carcinogen-induced tumour in the parent strain and various F_1 hybrids. By using congenic mouse strains the increased survival times could be related to the H-2 type of the host, some F_1 hybrids exhibiting resistance and some not. The results indicated that in this system a single IR gene was inadequate to explain the hybrid resistance and it was suggested that interaction of more than one H-2 genes was possible. It was also demonstrated that F_1 mice identical at the H-2 locus but different at other loci could also exhibit different degrees of

resistance, presumably of a non-immunological nature.

There is also evidence that connects the H-2 type to the antigenicity of tumours. It has been suggested that cytotoxic T-lymphocytes can recognise syngeneic H-2 determinants that have become "re-arranged" by virus infection (33) or chemical modification (34). Furthermore, the major histocompatibility complex determines susceptibility to cytotoxic T-cells directed against minor histocompatibility antigens (35). It is therefore tempting to suppose that tumours, at least those subject to T-cell control, might be recognised by such a mechanism. For virus-induced tumours there is reason to suppose this might be the case as it has recently been reported (36) that the H-2 region of the murine sarcoma virus-transformed target cell is important in <u>in vitro</u> T-cell cytotoxicity.

For carcinogen-induced tumours the evidence is contradictory. Oth <u>et al.</u> (37) induced a sarcoma in F_1 hybrid mice and two isoantigenic variants were selected by loss of one parental H-2 type. When the specificity of the tumour-associated transplantation antigens of these variants were compared in F_1 mice it was found that the transplantation antigens did not cross-react, thereby indicating that the loss of the H-2 antigen was associated with the loss of the tumour-specific antigen. An odd feature in this experiment was the finding that the original F_1 tumour was non-antigenic in the F_1 host whereas the variants were antigenic. Klein <u>et al.</u> (38), using somatic tumour cell hybrids and variants selected for loss of parental chromosome 17 (known to code for major histocompatibility complex), showed that loss of <u>either</u> parental chromosome resulted in loss of

antigenicity when tested in a homozygous parental strain. They concluded that the tumour-specific antigen was not localised in the major histocompatibility complex, but its expression was controlled by it. There may be some similarity between this mechanism and the control of T-cell recognition of minor histocompatibility antigens by major histocompatibility antigens which are on different chromosomes (vide supra).

The hypothetical anti-tumour mechanisms discussed above depend on T-cells and are subject to control by the major histocompatibility complex. There are, however, reports of non-T and non-B-cell killing of tumour cells across H-2 barriers. This activity would appear to be directed against a Moloney virus-induced antigen (39) and occurs naturally in certain strains of mice. It is known that in vitro killer activity and in vivo resistance correlate with the H-2 type of the host and it has been postulated that these effects are exerted through IR genes.

Another source of antigenicity in tumour cells is embryonic antigen. These antigens are putatively cross-reacting and although in some reports the relevant negative controls (with normal tissue target cells to exclude non-specific background noise) are absent, the existence of cross-reacting antigens seems beyond dispute. It has been suggested that tumour-specific transplantation antigens are of embryonic nature, normally not expressed in fully differentiated non-neoplastic tissue (40). However, the findings that lymph-node cells from multiparous rats (41) or mice (42) can kill a range of tumour types in vitro, and that transplantation antigens can be demonstrated

in vivo on cells that lack embryonic antigens in *in vitro* tests (43), indicate that cross-reacting embryonic antigens detected *in vitro* are different to tumour-specific transplantation antigens. This position was formally demonstrated by Fritze *et al.* (44) who showed that tumours raised with different chemicals, in the same strain of mice, did not cross-react in transplantation tests but the mice were able to produce antibody to the tumours which cross-reacted completely in complement-dependent *in vitro* antibody assays. In this experiment however, embryo cells could not absorb out the antibody activity.

Although *in vitro* detected cross-reacting embryonic antigens are separate from tumour-specific transplantation antigens, are they exploitable by the host to combat the tumour? The answer to this is complex as it depends on a) the genetically determined capability of different species and different strains to respond immunologically to foetal antigens; b) the quantitative expression of foetal antigens which can vary between tumours; c) the modulation that foetal antigens may undergo *in vivo* (43). Thus C57Bl mice, which are good responders to foetal antigens (43), can be immunised with foetal tissue against a syngeneic fibrosarcoma which has a large amount of foetal antigen on its surface, as detected *in vitro* (43, 45). Under less favourable circumstances foetal immunisation may be ineffective (46).

In addition to the factors discussed above, there is evidence that the mode of presentation to the host may influence the effectiveness of foetal immunisation. Thus, only X-irradiated foetal cells induced immunity in hamsters against SV-40 transformed cells; viable cells did

not (47). Furthermore, Forbes showed that the patterns of cross-reactivity when syngeneic tumours were used as immunogens with Freunds complete adjuvant were different to when they were used for challenge (48).

3. NATURE VERSUS NURTURE AS ETIOLOGICAL FACTORS IN HUMAN NEOPLASIA

The age old dispute between what Dobzhansky (49) has called the myths of Genetic Predestination and Tabula Rasa is apparently in the process of being reconciled by the following formulation. All individuals are bundles of particular potentialities which can be called forth, or left dormant, by the action of the environment. Medicine affords numerous examples of the interaction of genotype and environment. These include schizophrenia, haemophilia and, perhaps the best understood, phenylketonuria.

The study of phenylketonuria has been rendered considerably easier by the discovery of a genetic marker which is the cause of the disease. The physiological defect is a disturbance of the metabolism of the amino acid phenylalanine, accumulation of phenylalanine in the body fluids causing irreversible brain damage. Thus, phenylketonuria can be shown to be due to a recessive gene, untreated homozygotes suffering severe mental retardation. However, the environment of such individuals can act to free them from their genetic constraints. If the disease is diagnosed early enough its manifestations can be controlled by a diet nearly free of phenylalanine: diets coming assuredly from the environment.

The above example illustrates a principle and is not typical of all nature-nurture interactions. In practice it is not always possible to manipulate the action of the genes due to our present state of knowledge (or ignorance). Thus, in some conditions such as schizophrenia, which have given hints of a genetic component, there is no obvious genetic marker to give a handle for investigation, or the condition may be under polygenic control, which may be the same thing.

In oncology there may also be examples of the nature-nurture interactions. Thus, Möller has recently proposed a scheme in which oncogenicity is linked to mutagenicity; the carcinogen accelerating the appearance of rare genetic changes leading to neoplasia. The oncogenic event could take place by carcinogen binding to the DNA and initiating errors in replication, or possibly a somatic mutation could occur during repair of the DNA lesions caused by the carcinogen (50). It may be envisaged that the lesion induced by the carcinogen is random, leading to unique tumour antigens, whereas the oncogenic lesion of virus-transformed cells would be expected to take place at a specific point in the genome, leading to common antigens. Support for this proposition comes from the work of Fialkow (51) which indicated that the cells of many neoplasms were monoclonal by means of the isozyme glucose-6-phosphate dehydrogenase and immunoglobulin idiotype markers. This isozyme exists in allelic forms and is X-linked. Therefore, heterozygous women will express only one of the two alleles on each somatic cell, as only one random X-chromosome is expressed per cell. Thus, tumour cells expressing only

one marker demonstrate that a frank neoplasm is a monoclonal entity (although a nascent tumour is not necessarily so (52)).

If Möller's scheme is correct then, in a sense, cancer offers an example of nature-nurture interactions with the inimical influence, in the shape of the carcinogen, coming from the environment. It follows that this situation could be alleviated by identifying as many mutagens as possible in the environment, and promptly removing them. The former has been done for tobacco (53) but sociological and economic factors preclude the latter.

It would be incorrect to see cancer as a disease afflicting only post-industrial man. The evidence from fossil remains of bone sarcomata and other osseous tumours bears witness to the antiquity of cancer, and is not inconsistent with the view that cancer is coterminous with life (54). Although it may be true that cancer is evident in any environment, with the onset of the Industrial Revolution it became obvious that the milieu supporting life was also responsible to some extent for the incidence of cancer; some authorities putting the figure as high as 90% (55).

Are there then any cancers which can be assigned to a unifactorial genetic cause? The answer is yes, but the conditions as might be expected are rare (56): polyposis coli, a precancerous lesion of the large bowel, is inherited as an autosomal dominant trait; neurofibromatosis, in which tumours of the nervous system occur, is inherited as a dominant trait; and another example may be xeroderma pigmentosum, inherited as a recessive trait. In contrast with those conditions are the "industrial cancers" in which environmental

factors are of primary importance and heredity, as opposed to the material of inheritance (DNA), is apparently unimportant. It may be advisable, however, to treat the idea of environmental cancer with caution, in view of a possible predisposition to bronchogenic carcinoma because of the genetically determined differences in the ability of individuals to metabolise polycyclic hydrocarbons present in tobacco smoke (56).

If we imagine a continuum between the extremes discussed above, then most human neoplasms are probably caused by a mixture of genetic and environmental factors somewhere between these extremes. Most studies indicate that cancer is only very rarely inherited in a simple Mendelian fashion. What is often found is that the genetic element is modified, presumably by other genes or environmental influences. The techniques used to show this modulated genetic influence in cancer susceptibility are family studies, blood group studies and epidemiological studies. Family studies show that the incidence of cancer among the relatives of cancer patients was doubled when compared to the incidence in control populations: from 5% in the case of breast cancer and 1·5% in the case of stomach cancer (56). Twin studies (56) showed that for cancer of the breast and cancer of the uterus concordance was only slightly higher in identical twins, although in stomach cancer 3 out of 11 identical twins were concordant.

It is generally recognised that persons of blood group A are more likely to develop stomach cancer (and pernicious anaemia) than persons of groups O or B (57). No association between the blood groups and cancers of the lung, breast, colon and rectum have been found.

Epidemiological studies on Japanese migrants to the USA (58) showed that the high gastric cancer rates seen in Japan also occured in the immigrants; the rates for Japanese born in the USA were however much lower than for their parents, although still greater than for American whites. Conversely, for cancer of the large intestine the rate in immigrants rose rapidly from the low level prevailing in Japan to that of American whites. In contrast to these demonstrations of environmental influence it was found that migration failed to raise the low breast cancer rates found in Japanese women.

It should be noted that despite the implication of genetic factors in cancer, there is no evidence from the cytological studies carried out so far that specific changes at the chromosome level lead to neoplasia (59).

4. ESCAPE FROM IMMUNOSURVEILLANCE

If we take surveillance of cells transformed by various ubiquitous viruses as an established fact, then two questions become important: a) is the surveillance of transformed cells distinct from antiviral immunity? b) how do transformed cells escape from surveillance?

R.T. Prehn (60) has stated that it is not always clear whether the immune response is active against virus-transformed cells or the virus itself. However, if we consider the models discussed above we see that for Marek's disease there is no difference in virus shedding between infected resistant or infected susceptible chicks, or between untreated and vaccinated susceptible chicks; in spite of large differences in tumour incidence (61). In polyoma oncogenesis

antiviral immunity, induced by passive transfer of antibody or killed viral vaccines, does not protect against the outgrowth of transformed cells. However, immunisation against polyoma-induced cellular antigens does, in the absence of antiviral antibody (62). Furthermore, there is evidence in monkeys and man that the levels of antibody to H. saimiri and EBV antigens respectively are not directly related to the tumour incidence (10). In the case of feline leukemia virus (FeLV) the situation is the reverse of polyoma oncogenesis, FeLV-transformed cells being recognised by a viral envelope antigen on their surface (15).

The central question of tumour immunology is whether, at some stage, the proliferation of a nidus of malignant cells in humans is capable of being checked by immune surveillance. One might expect neomorts would afford excellent substrates for such experiments; however, more investigation seems to have been done on live subjects. In a scientifically correct but ethically dubious study Brunschwig et al. (63) showed that large numbers of autotransplanted cells were necessary before a take could be achieved. An optimistic view would be that this was due to an immune reaction.

Another study examined the fate of tumour cells inadvertantly transplanted with a renal allograft (64). In two cases it could be unequivocally shown that recipients on immunosuppressive therapy, with distant metastases, could eradicate tumour deposits if the primary tumour (transplanted kidney) was removed and immunosuppression was discontinued. It should be noted that discontinuation of immunosuppression alone was inadequate. Although the malignancies were rejected

as allografts the histocompatibility match must have been good as the kidneys had achieved normal function at 18 and 36 months, albeit under immunosuppression.

Although immunosurveillance against nascent tumours appears to be a perfect example of the maxim that "yesterday's truths become today's special cases", considerable ingenuity has been expanded in formulating escape mechanisms, some of which have taken precedence at various stages in the evolution of tumour immunology. The following list is taken from Klein (62):

a) Inadequate recognition of tumour antigens (lack of antigenicity of tumour)

b) Immunoresistance

c) General immunodeficiency of the host

d) "Sneaking through"

e) Abberation in the immune response.

a) Inadequate recognition of tumour antigens

It does not follow that because a tumour is not antigenic in rejection tests that its cell membrane is indistinguishable from that of a normal cell. Indeed, if contact inhibition is taken as a measure of the difference between malignant and normal cells then all malignant cell lines are different. What it does mean is that the tumour antigens are not recognised by hosts of a given genetic constitution; examples of this for virus-and carcinogen-induced tumours have been discussed in the section on surveillance. Although most of the common virally induced tumours are well recognised, it would appear that the carcinogen itself is important in determining

whether carcinogen-induced tumours are recognised in the syngeneic host. Thus, methylcholanthrene and dimethylaminoazobenzene elicit well recognised tumours in the syngeneic host, whereas acetylamino-fluorene-induced tumours are not detectably antigenic in rejection tests (65).

An analysis of the data on lymphocyte killing of tumour cells in vitro would seem to allow the following formulation: animal tumours possess unique antigens which elicit rejection responses, and common embryonic antigens which in general do not. Human tumours may possess tumour antigens which are peculiar to the histological tumour type (30). Baldwin has speculated that cross-reacting antigens detected in vitro, in both humans and animals, may be of similar significance (66). If this is so, previous attempts at prophylaxis with embryonic tissue in animal models do not hold out much hope as they have met with only limited success (43). Interestingly, in mice, the rejection of a syngeneic tumour transplant after embryo immunisation seems to occur only in mice with a C57Bl genetic background.

Reasons for the inefficiency of embryonic antigens as transplantation antigens have been postulated. Baldwin has discussed the possibility that tumour-associated embryonic antigens are expressed transiently on the cell surface, due to intracellular location or feeble attachment to the plasma membrane (41). Klein has suggested (10) it may be because tissue differentiation antigen expression is modulated in vivo by antibody, via mechanisms akin to the thymus - leukemia antigen system (67). Indeed, Lappe (68)

contends that a crucial function of the immune system is to "orchestrate" tissue development in the embryo and keep cell types at an appropriate level of differentiation. Thus tumour immunology is subsumed under this general heading.

b) Immunoresistance

Variability arises in a tumour cell population in an apparently random, genetically stable, clonal manner, as evidenced by the progression from solid to ascites tumour (69). When a selective pressure is exerted on these variants by an immune reaction against a specific antigen, cells with reduced amounts of the surface antigen will grow preferentially (70). As the selection exerted by the immune system is via the cell phenotype and not the mechanism by which it arose, it might be expected that, like drug resistance, immunoresistance can have a variety of underlying mechanisms. In two immunoresistant tumour lines studied by somatic cell hyrbidisation (10), it was found that the Ehrlich ascites tumour suppressed cell antigens on its partner cell and that these reappeared after chromosome loss from the hybrid, suggesting that the Ehrlich cell suppresses antigenic expression in a dominant fashion. Conversely another immunoresistant, H-2 antigen deficient, tumour line regained its antigen complement on hybridisation with a normal cell line.

c) Immunodeficiency of the host

Studies on the incidence of neoplasia in "nude" mice and in immunodeficient human subjects show some agreement. Thus, in "nude" mice reared under germ-free conditions a high degree of spontaneous

lymphoreticular neoplasms was noted in homozygous mice and a virtual absence in their haired heterozygous littermates (conversely the incidence of carcinogen-induced fibrosarcomas was similar (71)). In humans there is no doubt that primary immunodeficiency states favour development of leukemias and lymphomas over other malignancies. Indeed, each form of primary immunodeficiency seems to be associated with its own constellation of malignancies (72). Furthermore, in an analysis of 6,297 immunosuppressed transplant patients the risk of reticulum cell sarcoma development in men was 350 times greater than expected, whereas in women breast cancer was not increased (73).

As the increased incidence of lymphoreticular tumours is not in complete accordance with the surveillance theory, other explanations have been advanced:-

i) Chronic antigen stimulation accompanied by lack of suppressor activity. However, it should be noted that no difference in observed and expected incidence of lymphoreticular tumours was observed in leprosy patients, who are subject to immunodepression and chronic antigen stimulation (74).

ii) Infection with lymphocytotropic herpes virus due to immunosuppression.

iii) Oncogenic effect of immunodepressant.

d) "Sneaking through"

This term graphically describes the situation when a small number of tumour cells will "take", when a larger number is rejected, and a larger number still will overwhelm the host defences and also "take". The factors involved here, such as the manner of antigen presentation,

are of paramount importance in defining suitable immunotherapy regimes. What this mechanism demonstrates is that the animals inoculated with small numbers of tumour cells are not tolerant of the tumour antigen, but that their immune system is inefficient and must be mobilised in an appropriate manner.

e) Abberation in the immune response

This mechanism can be envisaged as operating at the level of the target or effector cell. Thus, free tumour antigen may "blindfold" the effector cells; tumour-specific antibody may impede recognition of target cell antigen; or antigen-antibody complexes may do both. However, if as seems likely, classical immunosurveillance is operative only in a limited context and that for the most part nascent tumours are unrecognised, then it follows that specific antibody can play no part in an escape from surveillance in the majority of cases. This is not to deny however that antigen alone may promote escape of nascent tumours in the microenvironment, or that antigen, antibody, or complexes may play some part at a later stage in tumour development. At this time the concensus seems to be that the serum of animals bearing progressing tumours contains blocking antigen-antibody complexes, whereas when the tumour is removed the serum contains "unblocking" free antibody (65).

These results were obtained using in vitro systems and their general in vivo relevance is questionable. It is, however, of interest that the systems in which "blocking" and "unblocking" factors appear to operate in vivo (75) are the very ones in which immunosurveillance may be a reality, i.e. polyoma-and Moloney virus-induced tumours in mice.

5. EFFECTOR MECHANISMS IN THE IMMUNE RESPONSE TO TUMOUR ANTIGENS

To sum up thus far: there appears to be an immune response mounted in the host to a variety of tumours. As a broad generalisation the response is of early onset in some virally induced tumours (where it corresponds to classical immunosurveillance) and of late onset in some carcinogen-induced tumours. It remains open to investigation whether tumours, that are not detectably antigenic at all in syngneic systems, can be rendered so by experimental manipulation. On this last point the future of immunotherapy, as a credible alternative to existing cancer treatments, may rest.

a) T-cell response

Although the debt of tumour immunology to transplantation immunology is great it may not prove to be totally beneficial. One possible pernicious influence is the idea that T-cells are the sole immunological mediators of an anti-tumour effect in syngeneic systems. However the central role of cytotoxic T-lymphocytes can readily be demonstrated in allogeneic combinations. Thus, Freedman *et al.* (76) showed that a tumour allograft of DBA/2 origin was rejected in irradiated C_3H mice reconstituted with immune spleen cells, but not with normal spleen cells. They furthermore showed that anti-θ treated immune spleen cells were ineffective but appropriately sensitised thymocytes were, and not surprisingly concluded that the rejection was due to T-cells. In various *in vitro* systems the cytotoxic effect of T-lymphocytes against allogeneic cells can be blocked specifically by antigen, antibody, or antigen-antibody

complexes (77); central and peripheral blocks also seem to exist in vivo (78).

There is good evidence in mice that sub-sets of T-lymphocytes are able to "scan" the H-2 regions of autologous cells, and can be triggered by a "rearrangement" of the H-2 antigens into differentiation to helper T-cells or cytotoxic T-cells. A rearranged H-2 type could be an allogeneic cell (79); a virus-infected cell (33); a chemically modified cell (34); or a lymphoid/reticuloendothelial cell with antigen on its surface (80). The production of cytotoxic T-cells against allogeneic cells, virus-infected cells, and chemically modified cells depends critically on the "re-arranged" H-2 K and D regions, whereas the production of helper T-cells is dependent on differences in the IR region (81). It may be of considerable significance for cellular co-operation in the immune response that IR gene associated antigens (Ia antigens) are expressed on most macrophages and B-cells, but only on sub-sets of T-cells. That is to say a sub-set of T-cells may "scan" Ia antigens on autologous macrophages and B-cells for "re-arrangement" due to antigen, before being "triggered" into proliferation to helper cells.

b) Non T-cell response

Certain it is that the remarkable chromosomal region called the Major Histocompatibility Complex is a unifying concept which explains many diverse immunological phenomena. However not all immunological reactions fall within its orbit. For instance sensitisation against xenogeneic tissue can be at least partially independent of T-cells (82). Thus, immunity against the DBA

mastocytoma is T-cell-dependent in allogeneic mice but antibody dependent in rats (83). Also "nude" mice are notoriously free of metastases after transplantation of xenogeneic tumour (9). Furthermore, immunological activity against syngeneic tumour antigens may (36) or may not (84) reside in T-cells.

One mechanism of non-T-cell killing is antibody-dependent lymphocyte cytotoxicity (ADLC). There is some debate as to the characteristics of the effector cells and antibody involved, but what is not in doubt is that specific antibody and Fc-receptor bearing effector cells are necessary. Some of the confusion may arise because the source of antibody in in vitro assays may be either extraneous, or from immune B-cells in the effector cell population (85). In the Moloney sarcoma/leukemia virus system, which has perhaps been the most studied, it would appear that antibody activity capable of rendering normal lymphocytes cytotoxic in vitro to Moloney antigen bearing tumour cells is present in progressor and regressor serum. However in the absence of antibody progressor lymphocytes, compared to regressor lymphocytes or lymphocytes tested before tumour development, were unreactive towards this antigen (85). It should however be noted that autochthonous combinations of serum and lymphocytes from progressor and regressor animals gave similar levels of ADLC (86).

In contrast, using the same system but different assay techniques, Harada et al. (87) found antibody capable of activating normal lymphocytes in vitro in regressor serum only. These authors also showed an excellent correlation between high levels of

serum antibody (as measured by immunofluorescence) to the Moloney antigen and regression of the tumour. The kinetics of antibody formation in this system seem to parallel those in a complement-dependent antibody system (88). Thus, Tamerius et al. found that antibody to Moloney antigen in regressor mice was present more often, and in higher titre, than in progressor animals.

In the non-syngeneic systems the ADLC antibody is generally found to be IgG (83), although as yet it is unclear whether one or all of the antibody classes and sub-classes is active in ADLC against a syngeneic tumour. There would seem, however, to be no doubt that passive transfer of antiserum with a high titre of antibody to the Moloney-determined tumour antigen can be effective against Moloney virus-induced sarcomas and lymphomas in vivo (89), although it is noteworthy that regressions depend on an intact thymus (90).

The antigen in question in the Moloney sarcoma/leukemia system is the Moloney leukemia virus-determined antigen which is present on transformed cells producing MSV and MLV (91). Transformed cells producing only MSV (91), or no virus at all (92), have been found to be "non-antigenic". However, after superinfection with MLV the antigenicity is recovered. It is interesting in this respect to note that a non-producer Moloney sarcoma virus-transformed line may be antigenic in F_1 hybrids (93).

Another immunological cytotoxic mechanism, also directed against the Moloney leukemia virus-induced antigen, has recently been described (39). It appears to be different to the classical

immunological mechanisms in that cytotoxicity is not dependent on an intact thymus or specific antibody; also, the effector cell has no B-cell markers and appears in certain strains of ostensibly unimmunised mice (94). It is also different from *in vitro* T-cell killing in that the effector cell can kill non-histocompatible target cells (39). Using high responder (R) and low responder (L) strains a genetic analysis, involving (R x L)F_1 xL back-cross mice, showed that *in vivo* and *in vitro* resistance to Moloney virus-induced lymphoma cells was H-2 dependent (95).

c) Macrophage response

There is evidence from concomitant immunity studies that animals bearing chemically induced (96), spontaneous (97), and weakly antigenic (31) animal tumours have a degree of resistance to further tumour transplantation. It is interesting that animals bearing a primary tumour appear to be imperfectly immunized against it; however, it can be rendered immunogenic in the autochthonous host by excising the primary tumour (98), or in syngeneic hosts by X-irradiated cells (99). Conversely, using transplanted tumours Fisher *et al.* (100) have shown that the degree of immunity is identical in rats whether or not they are deprived of their tumour burden. The first phase of concomitant immunity is specific but the second phase, as the tumour enlarges, is not (96). Cell transfer studies support the theory that macrophages participate in one or both of these mechanisms (101).

Using various criteria numerous authors report infiltrating macrophages in a growing, solid neoplasm, although not all are phago-

cytic (102). The macrophage content is similar for primary and transplanted tumours (103) and correlates directly with the immunogenicity and inversely with ability of the tumour to metastasize. In addition it is generally thought that macrophages, unless non-specifically stimulated, need lymphoid cells or cell products to exert an anti-tumour effect. Thus they can be activated *in vitro* and *in vivo* to kill target cell monolayers in a specific manner. Furthermore, it has been reported that macrophages derived from antigenic solid tumours are specifically active *in vitro* against tumour cells; however, macrophages from a non-antigenic tumour were not (104).

Support for co-operation between cells in control of tumour growth comes from the observation that tumour cell populations, deprived of macrophages by trypsin-resistant plastic adherence, produced metastases when injected into syngeneic hosts. However, tumour cell populations deprived of all infiltrating host elements did not metastasize (103). Cell co-operation in combating human neoplasia appears a reasonable proposition when it is considered that some breast tumours contain 10 - 30% macrophages (105), and that sinus histocytosis and follicular hyperplasia are associated with a good prognosis in breast cancer (5).

d) Antibody responses

In the early days of contemporary tumour immunology Görer (106) emphasized that isoantibodies could act synergistically or antagonistically with lymphoid cells. Another pioneer, Prehn, has consistently argued that an incipient immune response is harmful and promotes tumour growth, whereas a full-blown response is beneficial

(8). It would appear that Prehn is thinking of cellular enhancement mechanisms, although there have been occasional reports of enhancement of tumour growth in syngeneic systems after passive transfer of anti-tumour serum. Thus, virus-induced (107) and chemically induced (908) tumour growth could be enhanced, although it was emphasised in the only report to give data from repeat experiments (109) that the phenomenon was variable for a carcinogen-induced tumour.

The enhancing antibody is usually held to be 7S by analogy with allograft enhancement (78) or *in vitro* blocking activity (110). It is generally found to be cytotoxic to tumour cells *in vitro*; however, it has recently been demonstrated that IgM alloantibody is cytotoxic *in vitro* only to target cells with a high antigen density and it can enhance renal allografts which have a low surface antigen density (111). In the enhancement of carcinogen-induced tumours discussed above there was generally some specificity observed. Conversely, Goldberg *et al.* showed that dramatic enhancement of a chemically induced sarcoma could be achieved with a serum raised against syngeneic or allogeneic foetal antigens (112).

The enhancing effect of antibody in syngeneic tumour systems could be via free antibody or antigen-antibody complexes. Free antibody is unfashionable at the moment and complexes are in vogue, although few reports seem to have tested *in vivo* whether they are a cause or an effect of a growing tumour. It would also be of interest to know the incidence of antibody deposits in the kidneys of animals or patients with large tumours.

There is no evidence that antibody is effective against solid tumours and indeed it may occasionally enhance their growth. With regard to disseminated intravascular tumour cells (leukemia and metastatic cells) the situation may be different. Thus, using suitably absorbed xeno- or allo-antisera a protective effect against syngeneic lymphomata could be obtained (113). Tumours that do not metastasize in the syngeneic host can be induced to do so by immunosuppressive treatments such as ALS (114), thoracic duct drainage, and thymectomy plus X-irradiation (115). There is a correlation between the ability of a tumour to metastasize and its macrophage content (115), and it has been shown that tumour cell populations selectively depleted of macrophages may metastasize (103).

It is of considerable interest that tumour cells depleted of macrophages by continuous culture do not metastasize (103), suggesting the growth regulatory role of macrophages is itself influenced by humoral factors and/or cellular elements which are removed by continuous culture. Proctor et al. (116) have also detected an immunologically specific humoral factor which prevents the occurrence of lung metastases in rats with localised tumours. Prehn's group have recently confirmed earlier data, which suggested the presence of an antibody specific for autochthonous human melanoma cells in the sera of patients with localised disease (117). Patients with metastatic spread did not possess this antibody activity. In a similar human system it has been postulated that tumour-specific antibody activity may be depressed due to a specific

antibody against the tumour-specific antibody, and not due to antigen-antibody complexes as generally supposed (118).

It will be obvious from the above that antibody alone, or in conjunction with cells, is potentially capable of mediating numerous effects concerned with the growth of tumours. This however is catered for in the polymorphism exhibited by antibody classes and sub-classes.

6. AUGMENTATION OF IMMUNE RESPONSE TO TUMOUR ANTIGENS

It may not be completely illusory to say that all tumours have antigens (65), although not necessarily recognised or capable of mediating tumour rejection (44). The central problem may then be to promote a rejection reaction in the host against such antigens, that is to overcome immunological unresponsiveness. As yet there is no agreement as to what factors, or combination of factors, constitutes a rejection reaction. However, immunological theory offers at least two ways in which unresponsiveness may be overcome. These are a) the action of adjuvants such as C.parvum and BCG; b) by coupling the unrecognised antigen to a carrier that can be recognised by the host, for example by chemical modification of the tumour cell surface or by the use of somatic cell hybrids.

a) Action of adjuvants

The principles of tumour immunology could have been vindicated by the unequivocal success of immunotherapy. Such success, however, has not attended efforts to passively transfer immunity, and active immunisation seems most "effective" when tumour antigen is injected together with adjuvant. The most widely used adjuvant in clinical

trials has been BCG, and with some reservations about retrospective controls in clinical trials, it would appear that it is beneficial to some extent in the treatment of human neoplasia (119). Thus, Mathé et al. showed that in acute lymphoid leukaemia patients brought into remission with chemotherapy and maintained on BCG plus allogeneic leukemia cells, there have been no relapses in 4 years. This is in marked contrast to what usually occurs with patients on maintenance chemotherapy. Gutterman et al. reported similar findings in adult acute leukemia patients using BCG only. In contrast, Russell et al. using a different BCG to the above authors reported that acute myelogenous leukemia patients in remission all relapsed, whether treated with chemotherapy or chemotherapy plus immunotherapy (BCG plus allogeneic cells). The most noteworthy fact about this study was the markedly increased survival time of the immunotherapy group after relapse compared to the chemotherapy only group.

There are a number of reasons which would account for the differences in the results of Mathé et al. and Russell et al. The disease is different and in acute myelogenous leukemia the prognosis is generally worse than acute lymphoid leukemia. In addition, the Glaxo BCG used in the unsuccessful trial has been shown to be considerably less efficient in animal tumour models than the Pasteur BCG used in the successful trial (119).

For tumours in which an objective minimal residual disease situation is hard to attain, some clinical improvement has also been reported after adjuvant therapy. Thus, Morton et al. report that intralesional BCG is "a most effective treatment" for localised

melanoma and in addition may have a small effect in terms of recurrence and survival rates, as an adjunct to surgery in Stage II and III melanoma patients. Gutterman et al. report that in disseminated breast cancer patients brought into partial remission with chemotherapy, remission and survival are prolonged on subsequent treatment with chemotherapy plus BCG compared with chemotherapy alone (119).

The literature on the clinical use of C.parvum as an anti-cancer agent in man is not as extensive as for BCG, although in animal models it would seem to be as effective as BCG. There is impressive agreement as to the modes of action of C.parvum as an anti-cancer agent in experimental models (120, 121, 122). Administered systemically (i.p. or i.v.) it exerts a predominantly thymus-independent anti-tumour effect in which growth of tumours is inhibited in treated animals but no regressions occur. When administered intralesionally or mixed with irradiated tumour cells the effect is specific, T-cell-dependent, and results in frequent tumour regressions. Generally the systemic mechanism needs more C.parvum than the local injection, and it has recently been shown that in some cases presensitisation with C.parvum fails to modify the anti-tumour effects mediated by either mechanism (123). In addition to direct activation of macrophages and the potentiation of the tumour-specific response, C.parvum-immune T-cells may also activate macrophages indirectly (124) at a later stage than direct activation.

It might be envisaged that different permutations of these mechanisms, together with humoral mechanisms recently described

(125), would participate in any given situation depending on the tumor-host relationship. Thus, it has been shown that in two different tumour-host models the routes of C.parvum injection that gave optimum protection were different for each model. Also splenectomy abolished the protective effect of C.parvum in one model but not the other (126). More than one mechanism may well operate in the systemic anti-tumour effect because C.parvum, although still active in T-cell-deprived mice, is not as effective as in intact mice (127).

There is some suggestion that macrophages activated with high doses of C.parvum suppress T-cell killing in ^{51}Cr-release assays (128). A direct extrapolation to the in vivo situation would indicate that T-cell killing as such is unimportant in mediating the anti-tumour effects of C.parvum. Such conclusions however should be treated with reserve.

b) Activation of helper T-cells

It has been postulated that histocompatibility linked IR genes have a role in the recognition functions of T-lymphocytes, in some ways comparable to the immunoglobulin system (129). The general features of responses regulated by H-linked IR genes are: 1) The responses are T-cell-dependent in every case; there have not been H-linked IR genes identified that control antibody responses to T-independent antigens; 2) The IR genes control the carrier recognition function of T-cells, either in the development of helper T-cells for B-lymphocytes or in the elicitation of cell-mediated immune responses; 3) The absence of carrier function at the T-

cell level in an individual lacking a certain IR gene, and thereby preventing a response to an antigen, can be circumvented by linking that antigen to an immunogenic "carrier" molecule to which the individual is capable of responding at the T-cell level.

Attempts to overcome unresponsiveness to tumour antigens based on the above have not received a great deal of attention, although there are some indications that they have some chance of success. Jami and Ritz have shown that it is possible to immunise mice against syngeneic tumour cells by inoculation of somatic cell hybrids having such cells as one parent and allogeneic cells as the other parent. Furthermore, the hybrids expressed their tumour-specific antigens in pure bred mice even when they did not produce tumours in the F_1 hybrid (130). Chemical modification of tumour cells has also been reported to render them immunogenic in the syngeneic host. Thus, injection of mice with glutaraldehyde-(131) or trinitrophenol-(132) treated syngeneic tumour cells has been reported to result in rejection of a challenge with untreated viable tumour cells. In the first example, however, most of the activity could be ascribed to a non-specific inflammatory response to glutaraldehyde-treated cells.

7. PURPOSE OF THE PROJECT

During the last few years there has appeared some evidence, and considerable speculation, concerning the roles of lymphocytes (30), macrophages (133) and serum factors (30) in tumour-immune processes. It therefore seemed timely to examine a model of tumour immunity in

detail, and to dissect the mechanisms involved by means of *in vitro* assays of cellular and humoral immunity. The model chosen was the immunotherapy of a CBA mouse methylcholanthrene-induced fibrosarcoma, with the adjuvant *C.parvum*.

It was originally envisaged that the project would be divided into three separate, although ideally integrated, parts. The first part was concerned with extending the observation that *C.parvum* produced inhibition of tumour growth *in vivo*, and elucidating the mechanisms involved by the use of immunosuppressive procedures such as thymectomy and X-irradiation.

Part two was concerned with developing an *in vitro* assay of cellular immunity against syngeneic tumour cells. The technique chosen was the cytostatic assay, which utilises the *in vitro* uptake of a thymidine analogue by tumour cell monolayers to assess the "anti-tumour" activity of effector cells. After showing that the cytostatic cells in *C.parvum*-treated mice were macrophages, this work was concerned with showing a correlation between the *in vitro* and *in vivo* anti-tumour effects in *C.parvum*-treated mice, eg. in T-cell-deprived and X-irradiated mice; and also with examining the possibility that macrophages could be activated *in vitro* with lymphocyte products.

Part three of the project was concerned with examining the general humoral immune status of mice, either bearing a tumour or treated with *C.parvum*. This was done by assessing their ability to form antibody to defined antigens such as sheep red blood cells. Work was also undertaken to examine directly the humoral response to tumour elicited in mice treated with *C.parvum*. An isotopic anti-

globulin assay, which measured the *in vitro* uptake of mouse immunoglobulin onto target cells, was used for this. In addition, anti-*C.parvum* antibody and serum immunoglobulin class and sub-class levels were measured concurrently.

It was considered that an investigation of the involvement of immunoglobulin classes and sub-classes in tumour growth might help to reconcile the apparent differences between, for instance, blocking antibody, unblocking antibody, cytotoxic antibody, and antibody promoting cell-mediated cytolysis. As the complexity does not end with the above examples, but extends to the antigens against which the antibody is directed, and the mechanisms by which antibody is produced, these factors are discussed below.

a) Immunoglobulin classes and sub-classes and immune phenomena

While adjuvants are being increasingly used in the immunotherapy of tumours, there are few reports on their effect on immunoglobulin classes and sub-classes, or on the development of serum antibody which interacts with tumours. We felt that studies in this area were warranted for the following reasons:-

1) Adjuvants are known to have a variable effect upon the sub-class of antibody elicited in response to other antigens (134,135).

2) *C.parvum*, which is widely used in tumour immunotherapy, has been shown to exhibit a differential effect on IgG sub-class levels in man (136).

3) IgG sub-classes are known to differ widely in their *in vitro* and *in vivo* properties, including their ability to promote antibody-dependent cell-mediated cytotoxicity (137), and their ability to

fix complement and promote opsonisation (138).

4) There is preliminary evidence that the blocking activity which abrogates the cell-mediated destruction of tumour cells in vitro may be confined to certain sub-classes (139).

There is some debate as to whether C.parvum exerts its adjuvant action on T-cells or non-T-cells (ie. B-cells or macrophages). It has been suggested that C.parvum is primarily a B-cell adjuvant and in certain circumstances it can enhance the antibody response to type III pneumococcal polysaccharide (140). In addition it can enhance the antibody response to SRBC in experimentally produced 'B'-mice. Other indirect evidence that C.parvum acts primarily on B-cells comes from the observation that it affects the IgG_1 sub-class response the least. The significance of this is that the IgG_1 antibody response is thought to be the most thymus-dependent (141).

It would appear that to demonstrate the T-cell-independent adjuvant effect of C.parvum on the antibody response to SRBC the adjuvant must be given before the antigen. Thus, intact mice injected with C.parvum and type III pneumococcal polysaccharide simultaneously do not show an enhanced type III antibody response (142). Furthermore, "nude" mice injected simultaneously with C.parvum and SRBC failed to show an enhanced antibody response, although intact mice did under the same conditions (134). In addition to the dependence on the time of adjuvant administration with respect to antigen, the adjuvant effect of C.parvum may also depend on the dose, route of administration, and the physical nature of the antigen (134). This latter point may make comparison of results obtained with SRBC and

tumour cells rather difficult, although there are some parallels between the two systems as both seem to involve T-cell-dependent and T-cell-independent effects.

It has been observed that unless a given dose of antigen is itself large enough to elicit an immune response, an additional injection of adjuvant will not help to initiate such a response (135). Thus, a small dose of SRBC will elicit an IgM response but not an IgG_1 response; an injection of B.pertussis will increase the IgM response but does nothing for the IgG_1 response. However, once initiated the response can be radically changed; for instance an injection of adjuvant can make the difference between tolerance and specific antibody formation (143). The former result suggests that B.pertussis may act by mechanisms other than by effectively increasing the antigen dose; conversely, the latter is compatible with the notion that it does in fact do so. These phenomena may reflect T-cell-dependent and T-cell-independent mechanisms respectively. The T-cell-dependent activity may be due to the adjuvant stimulating T-cell proliferation (135), whereas the T-cell-independent activity may be due to an effectively increased antigen concentration presented to B-cells which therefore do not need helper T-cells. The latter could be via activated macrophages or antigen depot formation as is the case with Freund's complete adjuvant.

It is of interest that whereas C.parvum (134) and B.pertussis (135) enhance the IgG_2 response to SRBC, only the latter gives a comparable increase in the IgG_1 response to this antigen. Again this may reflect the fact that B.pertussis is more dependent than C.parvum on

T-cell-mediated mechanisms of adjuvant expression.

It has been a consistent, though little studied, observation that animals injected with Freund's complete adjuvant alone show a large increase in serum immunoglobulin. When an antigen is added to the adjuvant a large proportion of the extra δ-globulin is specifically precipitable by the antigen. Humphrey (144) has put forward the following possibilities for the presence of extra δ-globulin in the absence of added antigen: a) non-specific stimulation of antibody production against antigens which the animal has experienced in its past history; b) antibody against constituents of the adjuvant (eg. the tubercule bacilli); c) autoantibody against tissue components altered by the adjuvant; d) genuine non-specific δ-globulin produced by stimulating δ-globulin producing cells without this bearing the impress of a particular antigen.

In experiments to decide between these alternatives rabbits were injected with an antigen and when the titre of specific antibody subsided Freund's complete adjuvant was injected. It was observed that although the serum δ-globulin level rose the titre of antibody specific for the pre-injected antigen did not. In another experiment it was shown that when all of the antibody activity against the tubercule bacilli had been absorbed out there was still a considerable excess of δ-globulin unaccounted for. As it could also be shown that autoantibody against normal tissue was not raised after adjuvant injection it was concluded that the excess δ-globulin production was mainly due to a genuine non-specific or random mechanism (144).

The phenomenon seems to occur in other species, as injection of

C.parvum into humans (136) and mice (145) resulted in increases in serum IgG_2 and IgG_{2b} levels respectively. However these may not be directly comparable as mouse IgG_{2b} and human IgG_3 are thought to be homologous (138). In chickens elevated serum δ-globulin levels after adjuvant administration were found to be dependent upon granuloma formation at the site of injection and a delayed-type hypersensitivity response to bacterial constituents of the adjuvant (146).

The biological activities of immunoglobulins may be divided into two catagories: the specific reaction with antigen via the Fab portion of the molecule, and the consequences of antigen-antibddy reaction mediated via the Fc portion. Pleomorphism in the Fab region is of necessity almost limitless; however, structural variation in the Fc region also occurs on a more modest scale. This may indicate a corresponding range of functions for immunoglobulins in immune processes. Five classes of immunoglobulin have been defined in man on the basis of non-cross-reacting antigenic determinants on the Fc portion, and these have further been divided into so called sub-classes according to minor antigenic differences in the Fc region. As the sub-classes as well as the classes reflect a distinct amino acid sequence, the distinction between classes and sub-classes is rather arbitrary. This is particularly so in the mouse where the cross-reactivity between IgG sub-classes is less than for human IgG sub-classes (138).

As animal work is expected to have some relevance to humans, it is necessary to compare the immunoglobulin classes and sub-classes of man and mouse. Analogies between immunoglobulins of human and other

species are usually made according to physiochemical similarities or antigenic cross-reactions. Thus, IgG has the slowest electrophoretic mobility and a relatively low molecular weight; IgA forms polymers and binds to secretory component in the gastrointestinal tract; and IgM is a macroglobulin of about 900,000 Daltons. No direct relation between human and mammalian IgG sub-classes has been established, although IgG_1 and IgG_3 in humans are probably analogous to IgG_{2a} and IgG_{2b} in mice (138).

Of the phenomena that may be mediated by particular classes or sub-classes of immunoglobulin, that of *in vitro* blocking of cell-mediated cytotoxicity has been the most studied. There would seem to be no doubt, at least *in vitro*, that tumour-specific antibody combining with a critical amount of soluble tumour antigen can "block" cellular cytotoxicity against tumour cells (147); furthermore, antigen antibody complexes may "block" in *in vitro* models of immunological tolerance (148). The investigation of the nature of the antibody mediating the *in vitro* "blocking" effect is done by absorbing out a particular antibody class or sub-class and seeing if the "blocking" effect is still present in the antibody deficient serum. If it is found that only certain classes or sub-classes "block", and others do not, this could mean that the phenomenon is confined to these antibodies. Alternatively it could mean that the antibody that does not block has not been produced in response to the tumour antigen.

Using these methods it has been shown that the antibodies in multiparous mouse serum which "block" *in vitro* cell-mediated killing of methylcholanthrene-induced fibrosarcoma cells are of the IgG_{2a} and

IgG_{2b} sub-classes (149). In humans the *in vitro* "blocking" activity towards tumour cells from melanoma and colon carcinoma patients was absorbed out by *Staphylococcus aureus* which is known to bind selectively to human IgG_1, IgG_2 and IgG_4 (150). Furthermore the "blocking" activity against human neuroblastoma cells resided mainly in the IgG_1 and IgG_3 sub-classes (139). It is of interest that mouse IgG_{2a} and IgG_{2b} are thought to correspond to human IgG_1 and IgG_3 respectively (151).

As regards the antibody participating in antibody-dependent lymphocyte cytotoxicity (ADLC) *in vitro*, Pollack *et al.* (152) have shown in a syngeneic carcinogen-induced tumour system that it can be absorbed out of the serum of tumour bearing mice by an anti-mouse IgG_2 serum. However experiments with other specific antisera were not performed. In another syngeneic virus-induced tumour system both 19S and 7S serum fractions of regressor serum were able to promote ADLC *in vitro*. Furthermore in this system the IgM antibody appeared to be active at high concentrations and the IgG antibody at low (85). In humans the antibody participating in ADLC may be confined to the IgG class. Thus, serum from a choriocarcinoma patient could render suitable allogeneic target cells sensitive to ADLC and the antibody activity was confined to the 7S fraction of serum (153). Using the whole range of human class and sub-class myeloma proteins it was shown that only IgG_1 and IgG_3 inhibited ADLC in a xenogenic system using human effector cells and rabbit anti-target cell antibody. However, when the myeloma proteins were heat aggregated IgG_2 also became inhibitory (137).

b) Antigen against which "anti-tumour" antibody is directed

Antibody to syngeneic tumour cells has been demonstrated in the serum of experimental animals by either excising the tumour or rendering them immune to tumour challenge. A variety of techniques have been used such as the isotopic antiglobulin test (45), mixed haemabsorption (154) and complement-dependent lysis (155). The level of antibody observed is in general low by comparison with serum from animals immune to allogeneic or xenogeneic cells, and the function of the antibody *in vivo* is far from certain. It would appear that it is still to be unequivically shown, by extensive absorptions and use of a range of malignant and non-malignant cells, that the antibody response to a carcinogen-induced tumour can be directed towards tumour -specific transplantation antigens. On the contrary, syngeneic anti-tumour antibody is often found to be directed against embryonic antigens (45, 156) and is cross-reactive with other tumour cells (44).

Apart from antibody activity against unique and common tumour antigens there have been recent reports of naturally occuring antibody against a variety of tumour types (157), and shared antigens between bacteria and neoplastic cells (158). These observations may have the same underlying basis as there is evidence for the existence of a wide spectrum of environmental antigens capable of cross-reacting with tissue components of different mammalian species, including histocompatibility antigens (159). Rapaport and Lawrence have suggested that the need for immunity against bacterial, fungal and viral infections provided the selective pressures which culminated in an efficient thymus-dependent immune system. Presumably fortuitously, the cross-

reactions between microbes and mammalian tissue antigens produced the allograft reaction (159). In support of the theory these authors advance the arguments that the allograft reaction appears to be a secondary response and that certain bacterial antigens can induce allograft sensitivity in mammals. In addition the cross-reaction between mammalian heart tissue and certain strains of Group A streptococci might be considered (160).

c) Mechanisms by which antibody may influence tumour growth

Complement-dependent cytotoxicity has been extensively used to demonstrate antibody to tumour antigens. However, because this reaction generally needs heterologous complement (161) the *in vivo* implications are uncertain. It is of interest that the IgG sub-classes which most effectively bind complement (IgG_1 and IgG_3 in humans and IgG_{2a} in mice (138)) may be the very ones that mediate *in vitro* "blocking" of cellular cytotoxicity (149, 139).

Macrophages and monocytes are known to bind certain classes of antibody, either free or complexed with antigen. In man monocytes were shown to have receptors solely for IgG_1 and IgG_3; only these myeloma proteins inhibited rosette formation of IgG -coated heterologous red blood cells (138). This may mean that IgG_2 and IgG_4 are not cytophilic or opsonising; alternatively it could be that they are an insignificant part of the response to red blood cells. It also seems apparent that opsonising and cytophilic IgG_1 and IgG_3 antibodies bind at the same site. A direct examination of the binding between monocytes and IgG sub-classes by autoradiography revealed that only IgG_1 and IgG_3 bound strongly to the monocyte surface (162). The

binding could be inhibited by fresh serum or antigen-antibody complexes.

It therefore appears that IgG_2 and IgG_4 are not cytophilic, although they may bind to monocytes in the form of antigen-antibody complexes as it can be shown that heat-aggregated IgG_2 and IgG_4 do bind to monocytes. IgM failed to bind to monocytes when heat aggregated, and would only do so in its native form when complement was added (138). Thus, for the human IgG sub-classes the ability to fix complement correlates with the ability to fix to monocytes, and the available data from mouse studies is in agreement with this in that cytophilic activity has been shown to reside in the IgG_2 sub-classes. However IgG_1 also bound to mouse monocytes although with a lower binding affinity (163).

The relevance of antibody classes and sub-classes to "blocking" of in vitro cell-mediated cytotoxicity and antibody-dependent lymphocyte cytotoxicity has already been discussed, it therefore only remains to point out other, more speculative mechanisms of antibody involvement in tumour immune processes. Immunoregulation via receptors on the surface of cells of the immune system may be considered here. Mammalian cell membranes possess recognition sites that enable them to respond to various environmental stimuli. These are of interest in endocrinology, pharmacology and immunology. In the latter it is known that all lymphocytes bear receptors for antigen, but in addition certain subsets may have receptors for the Fc portion of immunoglobulin and also complement components (164).

The Fc-receptor appears to be ubiquitous in the immune system; thus, it is present on mast cells, neutrophils, monocytes, macrophages, platelets, B-lymphocytes and perhaps "activated" T-lymphocytes (165).

This is not to say that the Fc-receptors on all cells are the same; differences exist as to the avidity of interaction and the class or sub-class of antibody that is bound. Thus, the adherence of antigen-antibody complexes to lymphocytes was more stable than free antibody (166), whereas macrophages could bind antibody in the absence of antigen (162). Also the binding of mouse IgG_1 to lymphocytes is strong compared to the other classes and sub-classes (165), in marked contrast to macrophages which bind IgG_{2a} and IgG_{2b} most strongly (163).

The receptors for antigen, Fc portion of antibody, and complement would appear to be ideal mediators for immunoregulation. Indeed, it has been shown that feedback suppression of antibody formation by specific antibody is easier to achieve with whole antibody molecules, as opposed to $(Fab)_2$ fragments (165). Furthermore, it can be shown that delayed-type hypersensitivity reactions are promoted by procedures that suppress antibody formation (167). Thus a delayed-type hypersensitivity reaction to SRBC, which was known to be suppressed by antibody formation to SRBC, could be promoted by treatment with cyclophosphamide. The suppression of the reaction was due to complexes of antibody and antigen.

The phenomenon of immunoregulation may embrace the process of *in vitro* "blocking" of cell-mediated cytotoxicity by antigen-antibody complexes. In this respect the experiment describing the interaction of antibody formation and delayed-type hypersensitivity reactions may be relevant. In addition, it is known that enhancement of allogeneic tumour growth depends on the Fc portion of the enhancing antibody (168).

Another system of immunoregulation has been proposed by Jerne (169).

This theory depends on idiotypic determinants of the antibody being recognised by anti-idiotypic antibodies in the same animal. There is, therefore, a network of antibody molecules that recognise other antibody molecules, which in turn recognise still others. Since the patterns of variable region idiotypes are determined by the variable amino acid sequences of the polypeptide chains there are millions of different idiotypes. This is postulated to correspond to the vast range of antibody combining sites available in the immune system. Thus, lymphocytes are subject to continuous suppression by other lymphocytes and by antibody molecules, until introduction of antigen upsets the equilibrium and causes proliferation. However, the system will always tend to return to the original position of equilibrium.

Lewis et al. (118) have described a system in which a specific antibody against a tumour-specific antibody may be the "blocking" agent, rather than antigen-antibody complexes. In these experiments serum from a melanoma patient with, and without, tumour-specific antibody activity towards melanoma cells was used. It was shown by gel diffusion and passive haemagglutination that IgG from serum without tumour-specific antibody activity reacted with γ-globulin from serum that had this activity.

d) Plan of the project

From the foregoing it should readily be appreciated that antibody is potentially capable of mediating a vast range of activities which may influence tumour development. Therefore, what we have done is try to relate various serological parameters to models of tumour

immunity, principally the inhibition of tumour growth by C.parvum. The parameters chosen were: total immunoglobulin class and sub-class levels in serum; anti-C.parvum antibody; and immunoglobulin capable of binding to syngeneic tumour cells in vitro.

To achieve this aim antisera against mouse immunoglobulin classes and sub-classes were produced by standard techniques, and an indirect antiglobulin assay to detect immunoglobulin binding to tumour cells in vitro was developed. It was soon apparent that C.parvum elicited increased levels of immunoglobulin binding to tumour cells in vitro, and elevated levels of certain immunoglobulin sub-classes, concurrently with high anti-C.parvum antibody titres. Therefore, studies were carried out to ascertain their cellular basis and to compare the effects of different treatment schedules and different adjuvants. Furthermore, the specificity and characteristics of the immunoglobulin binding to tumour cells in vitro were examined, and an attempt was made to confirm the existence of "anti-tumour" antibody by a different technique. Finally, immunotherapeutic treatments based on the above in vitro results were attempted.

In addition, preliminary experiments were performed to investigate a) whether the CBA fibrosarcoma elicited a humoral response without the intervention of adjuvant; b) whether tumours of the lymphoid system could exert a general immunosuppressive effect on the humoral response to defined antigens, or influence K-cell activity.

MATERIALS AND METHODS

1) ANIMALS AND ANIMAL HANDLING

a) Mice

Syngeneic male and female mice of the A/HeJ ($H\text{-}2^a$), BALB/c ($H\text{-}2^d$) and CBA ($H\text{-}2^k$) strains were used in these studies. They were originally obtained from the MRC Laboratory Animal Centre, Carshalton, England. Since then they have been inbred for 12, 10, and 12 generations respectively in our own animal house, where they were housed in plastic boxes (5 - 6 to a box) lined with wood shavings. The temperature of the room was maintained at 22°C and a constant air exchange rate to provide adequate ventilation was ensured. Mice were maintained on a commercial mouse cake diet (McGregors of Leith, Edinburgh) and water.

Mice deficient in thymus-derived cells (T-cells) were obtained by thymectomy at 4 - 6 weeks, followed by whole body irradiation (850 rad.) one week later and reconstitution at that time with an i.v. injection of 4×10^6 anti-θ treated bone marrow cells. The mice were allowed to recuperate for 6 weeks before being used in an experiment.

The congenitally athymic (nude) mice used in one experiment were homozygous, and back crossed to a BALB/c background. They were either maintained at the Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia, or obtained from Bomholtgard Ltd., Denmark.

Mice injected with viable tumour cells in the foot were deprived of their tumour burden as follows. After mild ether inhalation

the tumour bearing leg was swabbed with 70% ethanol and the tumour excised at the knee joint with "bone crushers". A flap of skin was drawn over the thigh bone and sutured with cotton.

b) Rabbits

Adult New Zealand white female rabbits were used for the production of antisera to mouse proteins. They were kept in solitary confinement in metal cages and fed on a diet of rabbit pellets supplemented with greens.

c) Goats

The goats used for the production of anti-rabbit IgG were kept under standard conditions at the University Centre for Laboratory Animals (The Bush, Milton Bridge, Midlothian).

d) Guinea pigs

Guinea pigs, used as a source of complement, were kept under similar conditions to the rabbits.

e) Bleeding of laboratory animals

Mice were killed by ether inhalation and bled from the inferior vena cava.

Rabbits were secured in a suitable box with an ear protruding. After applying xylene to the ear a cut was made with a scalpel in the marginal vein and the blood collected. After stopping the flow by light pressure on the cut, the ear was swabbed thoroughly with ethyl alcohol to remove the xylene.

The goats were kindly bled by Dr. R.G. Dalton.

Freshly obtained blood samples were allowed to clot for 30 minutes at room temperature, rimmed and left overnight at $4^{\circ}C$.

The clot was removed and the serum spun at 1 - 2,000 r.p.m. for 10 minutes to remove remaining blood cells. Serum was stored at -20°C until needed.

2) PRODUCTION OF ANTISERA

a) Techniques for production of purified myeloma proteins

i) Ammonium sulphate precipitation. Mouse serum (1 vol.) was diluted with 60 mM phosphate buffer at physiological pH (3 vol.), then precipitated using 4M ammonium sulphate (4 vol.) added dropwise with constant stirring. The precipitate was harvested by centrifugation, redissolved in 60 mM phosphate (4 vol.) and reprecipitated with 4M ammonium sulphate as above. After further ammonium sulphate precipitation the globulin fraction was dissolved in 10 mM phosphate buffer (pH 7·5) and dialysed exhaustively against the starting buffer used for ion exchange chromatography.

ii) Ion exchange chromatography. The ion exchanger used in these studies was a microgranular pre-swollen anion exchanger on a cellulose matrix, DE52 (H. Reeve Angel & Co. Ltd., London). It was first equilibrated with a large volume of starting buffer, usually 10 mM phosphate (pH 7·5), and left overnight at room temperature. The supernatant was decanted off, the DE52 re-suspended in starting buffer and filtered through a sintered funnel using vacuum suction. After washing thoroughly with starting buffer the exchanger was resuspended in it, brought to the correct pH with HCl or NaOH, then degassed on a vacuum pump

before packing the column, After use the DE52 was regenerated in a large volume of 0·2M NaH_2PO_4 (pH 4·5), then re-equilibrated with 10 mM phosphate buffer (pH 7·5).

The labour involved in each separation was reduced to applying the sample and pooling the fractions, by the use of an LKB4701A Uvicord 1 to locate fractions of interest; LKB chopper bar recorder to record them; and an LKB7000 UltraRac to collect them. The mixing of solutions to form gradients or sequential steps was simplified by the use of an LKB11300 Ultragrad (LKB Instruments Ltd., L.K.B. House, 232 Addington Road, S. Croydon, Surrey). See Fig. 3 and Tables 3,5,6,7.

iii) Gel filtration. Sephadex G-200 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was suspended in a large excess of phosphate buffered saline (0·15M) and swollen by heating on a boiling water bath for 5 hours. To remove fines the swollen gel was allowed to settle and the supernatant removed. After a further cycle of suspension and decanting the gel was resuspended at the correct consistency, degassed and applied to the column. See Fig. 2 and Table 4.

iv) Starch block electrophoresis. Potato starch (600g) was washed three times with an excess of 0·05M barbitone buffer, poured on to the block and excess buffer allowed to drain away. The starch was left to solidify overnight then a well (6 cm x 1 cm) was cut in the centre of the block. To apply the sample (5 ml) it was mixed to a thick paste with potato starch and applied to the well. The block was then wrapped in

polythene and subjected to electrophoresis (300V, 35-40mA) for 28 hours at 4°C. At the end of the run the block was cut into 40 x 1 cm. slices and each slice suspended in PBS (20 ml) overnight. The protein content of each supernatant was estimated by the method of Lowry (170) and the suspensions pooled on the basis of this. Each pool was extracted with PBS (500 ml) and the extracts pressure dialysed to manageable volumes. See Fig. 2 and Table 4.

v) Enzyme digests.

Solution A - 8 mM EDTA, 20 mM cysteine-HCl in 0·2M phosphate buffer pH 7.

Solution B - Protein for digestion (30 - 50 mg in 5 ml of PBS) plus 0·5 or 1 mg of mercuripapain (Koch-Light Laboratories Ltd., Colnbrook, Bucks.).

Solution C - Iodoacetemide (0·8M) in 20mM phosphate pH7.

Solution A (5 ml) was added to solution B (5 ml) and left at 37°C for 4 - 5 hours. The reaction mixture was then made 20 mM in iodoacetemide to stop the reaction, left for 30 minutes at room temperature, and dialysed against 10 mM phosphate buffer pH 7·6. The fragments were separated on DE52 (see Fig. 4). It was noted that 0·5 mg of mercuripapain gave the better results when IgG_{2a} was digested, whereas 1·0 mg was better for IgG_1.

b) Injection of rabbits with purified myeloma proteins

New Zealand white rabbits (♀) were used for the production of antisera to mouse immunoglobulin classes and sub-classes. The

injection schedules are shown in Table 2.

c) Solid-phase immunoabsorption of rabbit antisera to mouse immunoglobulin classes and sub-classes

The columns for immunoabsorption were made using CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals) as follows. Sepharose 4B gel was swollen and washed for 15 minutes on a glass filter with excess 10^{-3}M HCl to remove dextran and lactose. The protein to be coupled was dissolved in coupling buffer (0·1M $NaHCO_3$ buffer containing 0·5M NaCl, pH8) at a concentration of approximately 2 mg/ml, mixed with the gel in a test tube and the mixture rotated end over end (see Table 8). Unbound material was washed away with coupling buffer and any remaining active groups were reacted with 1M ethanolamine at pH 8 for 1 - 2 hours. Three washing cycles were used to remove non-covalently absorbed protein, each cycle consisting of a wash at pH 4 (0·1M acetate buffer containing 1M NaCl) followed by a wash at pH 8 (0·1M borate buffer containing 1M NaCl). Finally the solid-phase immunoabsorbant was suspended in coupling buffer and poured into a suitable size syringe barrel. Further details of individual columns are given in Table 8.

Immunoabsorption of antiserum were carried out by concentrating the antiserum to a manageable volume (say 20 ml to 5 ml) then dialysing against coupling buffer. The concentrated antiserum was applied to the column and the progress of the serum constituents which did not absorb to the column monitored with the LKB4701A Uvicord 1. When all of the unabsorbed antiserum had been eluted the buffer was changed to one of a low pH (0·1M glycine/HCl containing 0·5M NaCl, pH ·2·8) to elute antibody that had been specifically retained on the

column. Under optimum conditions this retained antibody was visable as a discrete peak on the recorder.

The efficiency of immunoabsorbent columns prepared in this way is illustrated in Figs. 5 and 6.

d) Assessment of specificity of immunoabsorbed antisera

Crude antisera were first examined by immunoelectrophoresis against normal mouse serum, in order to obtain an idea of the immunoabsorptions necessary to render the antiserum specific (see Figs. 7 - 11). Thus we see that anti-IgM reacts faintly with mouse IgG_1; anti-IgA reacts with IgG, IgM and an unknown component in the α_2-region; anti-IgG_1 reacts with a slow γ-component (IgG_{2a}).

Gel diffusion analyses of anti-sub-class antisera were performed using a battery of purified myeloma proteins to confirm which immunoabsorptions were necessary: by this method it was found that anti-IgG_1 reacted with mouse IgG_{2a} as well as IgG_1 and that anti-IgG_{2a} reacted with mouse IgG_1 as well as IgG_{2a}. Table 9 shows the absorptions necessary before the antisera gave (1) single lines on immunoelectrophoresis against normal mouse serum, Figs. 7 - 11 ; (2) lines of identity with the homologous commercial antisera, Figs. 7 - 11; (3) no reaction against purified myeloma proteins not used in the production of that antiserum, Figs. 7 - 11.

As commercial standards became available the antisera were tested against these. It was again found that an antiserum only reacted against the protein that it had been raised against (Fig. 12).

e) Comments on production of antisera

While the production of antiserum is in general straight forward

TABLE 1 PLASMACYTOMAS USED IN THESE STUDIES

PLASMACYTOMA	IMMUNOGLOBULIN SECRETED	MYELOMA PROTEIN PURIFIED FROM	ORIGIN
MPC25	IgG_1	serum	Dr. S. Micklem, Dept. of Zoology, Kings Buildings, Edinburgh
ADJPC5	IgG_{2a}	serum	Dr. S. Micklem, Dept. of Zoology, Kings Buildings, Edinburgh
MOPC104E	IgM	serum	Dr. D. Dresser, Nat. Inst. Med. Res., Mill Hill, London
MOPC47A	IgA (one heavy + one light chain)	urine	Dr. S. Micklem, Dept. of Zoology, Kings Buildings, Edinburgh

TABLE 2 IMMUNISATION SCHEDULES FOR PRODUCTION OF ANTISERA TO MOUSE IMMUNOGLOBULIN CLASSES AND SUB-CLASSES

ANTISERUM RAISED AGAINST MOUSE	INJECTIONS			PROTEIN INJECTED	DAY WHEN BLED
	1 (a)	2 (a)	3 (b)		
IgM (MOPC 104E)	Day 0 2 mg S.C.	Day 25 2 mg S.C.	Day 45 2 mg I.P.	Whole molecule	Day 68
IgA (MOPC 47A)	Day 0 200 µg IM	Day 14 400 µg IM	Day 48 400 µg I.P.+S.C.	One heavy + one light chain of IgA molecule	Day 66
IgG_1 (MPC 25)	Day 0 1 mg S.C.	Day 19 1 mg S.C.	Day 41 1 mg I.P.	Fc	Day 66
IgG_{2a} (I) (ADJPC5)	Day 0 400 µg IM	Day 7 400 µg IM	Day 39 400 µg I.P.+S.C.	Fc	Day 48
IgG_{2a} (II) (ADJPC5)	Day 0 250 µg IM	Day 10 1·2 mg IM	Day 29 250 µg IP	Whole molecule	Day 36

a) Injection 1 and 2 in Freund's complete adjuvant.

b) Injection 3 in Alhydrogel (Dansk Svovlsyre - OG Superphosphate - Fabrik, Copenhagen)

TABLE 3 PURIFICATION OF IgA (ONE HEAVY + ONE LIGHT CHAIN)

STEP	PROCEDURE	COLUMN SIZE (cm) / AMOUNT OF SAMPLE APPLIED (OPTICAL DENSITY AT 280 mμ)	CONCENTRATIONS OF NaCl USED	CONCENTRATION AT WHICH RELEVANT PEAK ELUTED	COMMENTS
1	Fractionation of concentrated urine, from mice bearing MOPC 47A plasmacytoma, on DEAE cellulose.	30 x 2 / 14·4 (7 ml)	0,20,50,200 mM	Peak A, 200 mM	Peak A rich in IgA - recycle
2	G-200 separation of Peak A	60 x 2 / 6·0 (3 ml)	0·15 M	0·15 M	Peak smeared over 19S, 7S and 4·5S regions - IgA in ascending (B) and descending (C) portions. Immunise rabbit with B.

Note

1) All fractionations were carried out at 4°C with a flow rate of 10 - 20 ml/hr

2) All solutions of salt were buffered with 10 mM phosphate pH 7·6 for ion-exchange chromatography, and 60 mM phosphate pH 7·2 for gel filtration

3) Purity of fractions analysed by immunoelectrophoresis and gel diffusion.

TABLE 4 PURIFICATION OF IgM

STEP	PROCEDURE	COMMENTS
1	Separation of myeloma serum (22 ml) on G-200 sephadex	Select the 19S fraction
2	Recycle of 19S peak	
3	Separation of 19S peak into its components by starch block electrophoresis	Divide IgM peak into ascending and descending portions - both found to be pure IgM. Use each fraction for immunising a rabbit.

Note

1) G-200 runs were carried out at 22°C, in phosphate buffered saline pH 7·2, at a flow rate of 40 ml/hr, using a 100 x 4·4 cm column.

2) Purity of fractions analysed by immunoelectrophoresis and gel diffusion.

TABLE 5 PURIFICATION OF IgG_1

STEP	PROCEDURE	COLUMN SIZE (cm) / PROTEIN APPLIED (mg)	CONCENTRATIONS OF NaCl USED	CONCENTRATION AT WHICH RELEVANT PEAK ELUTED		COMMENTS
1	Separation on DEAE cellulose of fraction obtained from ammonium sulphate precipitation of pooled myeloma serum (16 ml)	30 x 2 / 360	0,40,100,150,200 mM	Peak A Peak B	100 mM 150 mM	Peaks A & B rich in IgG_1
2	Recycle of Peak A on DEAE cellulose	30 x 1·5 / 90	40,100,200 mM	Peak C Peak D	40 mM 100 mM	Peaks C & D pure IgG_1 - use for papain digest
3	Recycle of Peak B on DEAE cellulose	30 x 1·5 / 90	0,40,100,150,200 mM	Peak E	40 mM	Peak E pure IgG_1 - use for papain digest

Note

1) All fractionations on DEAE cellulose carried out at 4°C with a flow rate of 20 ml/hr

2) All solutions of salt were buffered with 10 mM phosphate pH 7·6

3) Purity of fractions analysed by immunoelectrophoresis and gel diffusion

TABLE 6 PURIFICATION OF IgG_{2a} (I)

STEP	PROCEDURE	COLUMN SIZE (cm) / PROTEIN APPLIED (mg)	CONCENTRATIONS OF NaCl USED	CONCENTRATION AT WHICH RELEVANT PEAK ELUTED	COMMENTS
1	Separation on DEAE cellulose of fraction obtained from the ammonium sulphate precipitation of pooled myeloma serum (27 ml)	60 x 2 / 800	0,40,100,150,200 mM	Peaks A & B, 40 - 100 mM	Rich in IgG_{2a}. Bread peak - split into ascending (Peak A), descending (Peak B) parts.
2	Recycle of Peak A on DEAE cellulose	30 x 2 / 225	20,50,75,100 mM	Peak C, 20 mM	Peak C - almost pure IgG_{2a} - use for papain digest
3	Recycle of Peak B on DEAE cellulose	60 x 2 / 150	20,50,100 mM	Peak D, 50 mM	Peak D - almost pure IgG_{2a} - use for papain digest

Note

1) All fractionations carried out at 4°C with a flow rate of 20 ml/hr
2) All solutions of salt were buffered with 10 mM phosphate pH 7·6
3) Purity of fractions analysed by immunoelectrophoresis and gel diffusion

TABLE 7 PURIFICATION OF IgG_{2a} (II)

STEP	PROCEDURE	COLUMN SIZE (cm) / AMOUNT OF SAMPLE APPLIED	CONCENTRATIONS OF NaCl USED	CONCENTRATION AT WHICH RELEVANT PEAK ELUTED	COMMENTS
1	G-200 Sephadex fractionation of myeloma serum	100 x 1·4 / 25 ml serum	0·15 M	Peak A, 0·15M	Obtain globulin fraction from 7S peak (A) by 4M ammonium sulphate precipitation
2	Separation of globulin fraction from step 1 on DEAE cellulose	60 x 2 / 320 mg	0,20,40,100,260 mM	Peak B, 40 mM	Peak B rich in IgG_{2a} - recycle
3	Fractionation of peak B on DEAE cellulose	30 x 1 / 45 mg	0,20,50,100,200 mM	Peak C, 50 mM	Peak C pure IgG_{2a} - used for injection of rabbit

Note

1) All fractionation on DEAE cellulose carried out at 4°C, with a flow rate of 20 ml/hr for the large and 5 ml/hr for the small column.

2) All solutions of salt used for ion exchange chromatography were buffered with 10 mM phosphate pH 7·6.

3) G-200 runs carried out at 22°C, in phosphate buffered saline pH 7·2, at a flow rate of 40 ml/hr, using a 100 x 4·4 cm column.

4) Purity of fractions analysed by immunoelectrophoresis and gel diffusion.

Fig. 2 PRODUCTION OF IgM

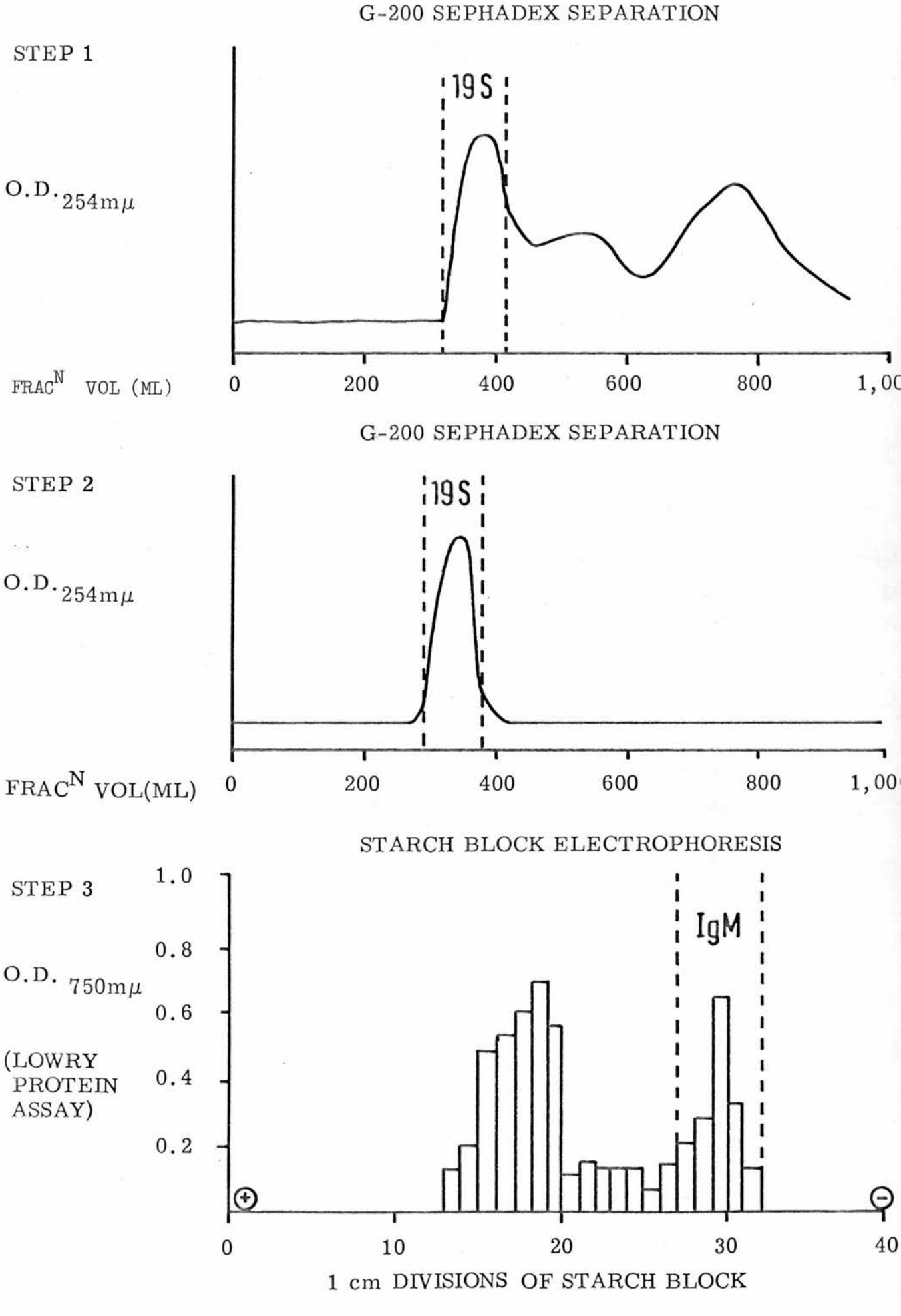


Fig. 3 PRODUCTION OF Fc FRAGMENT OF IgG_{2a}(I)

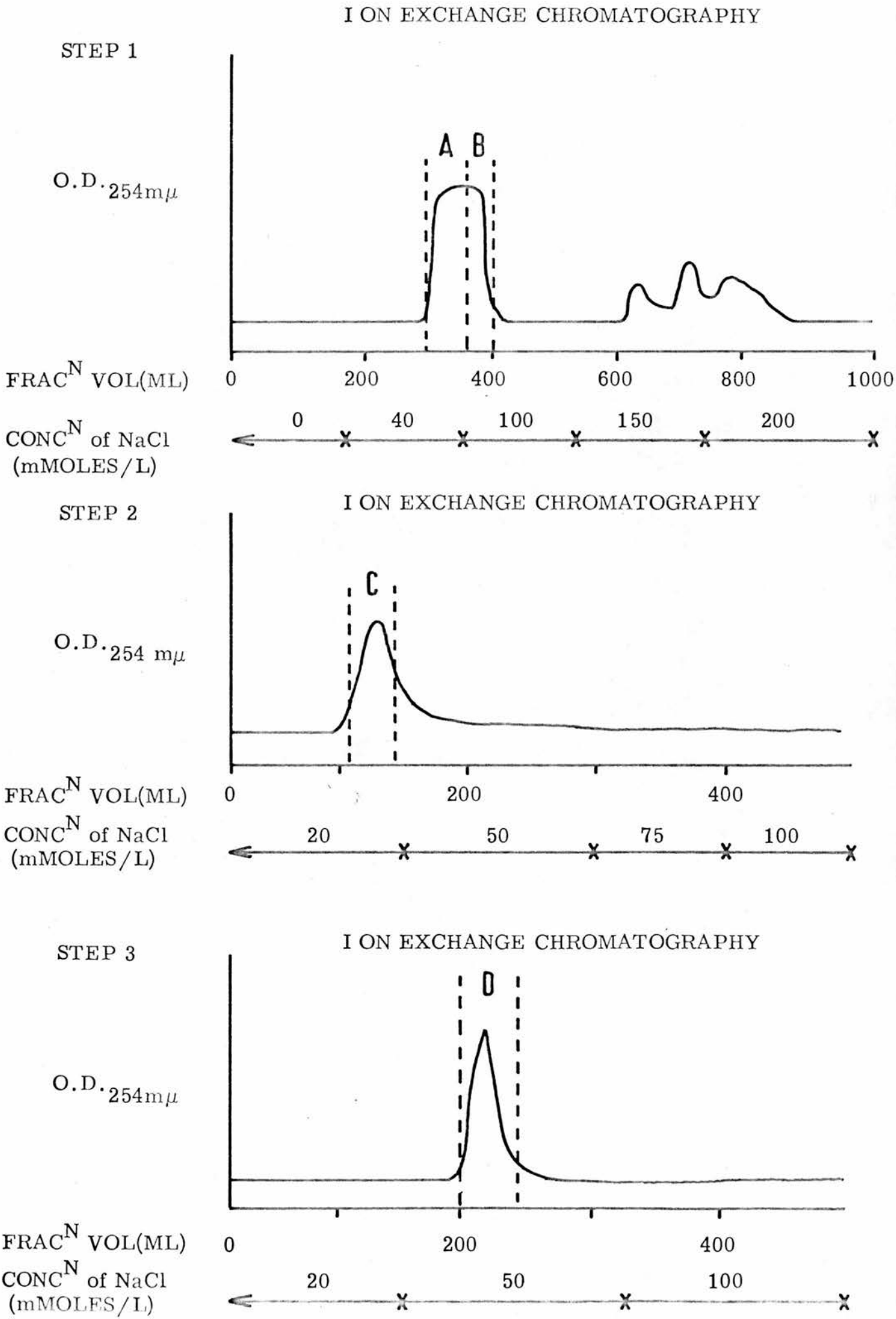


Fig. 4 SEPARATION OF PRODUCTS AFTER PAPAIN DIGESTION OF IgG_{2a}

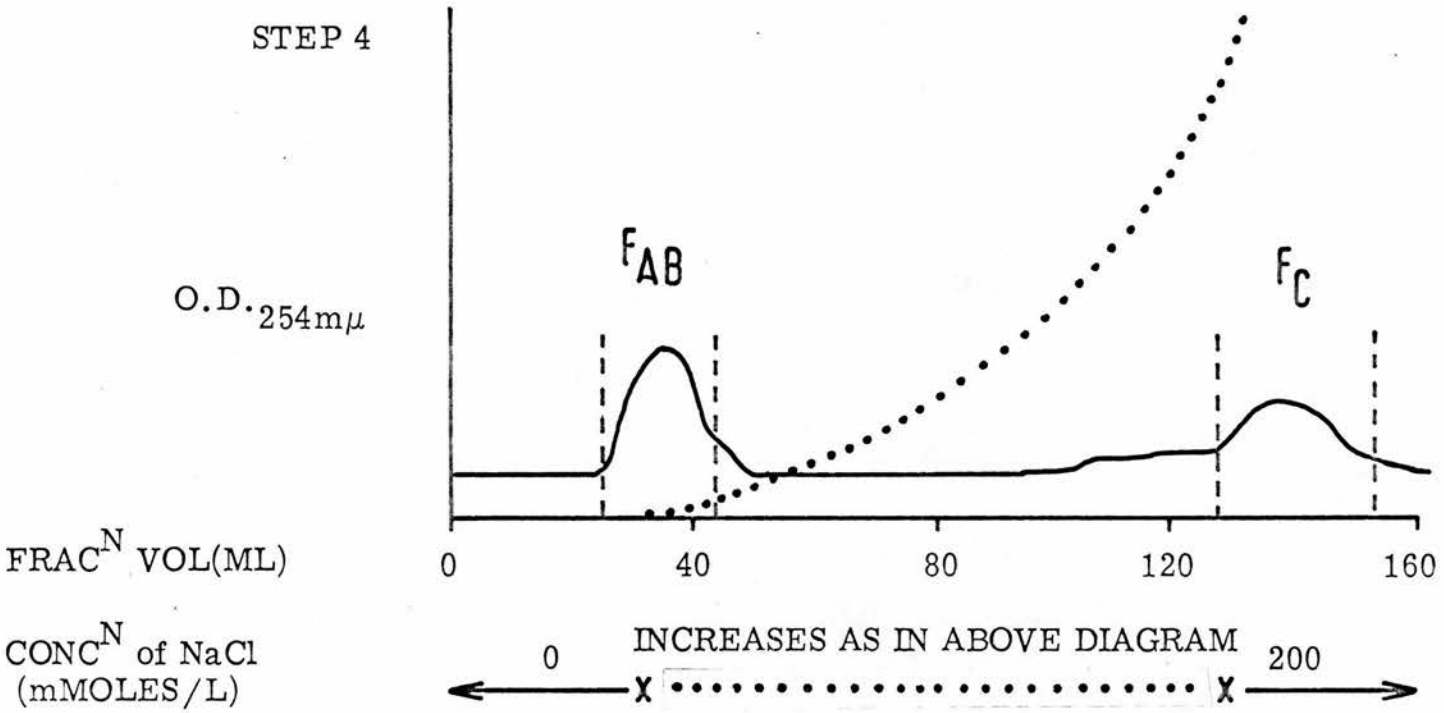


TABLE 8 IMMUNOABSORBENT COLUMNS

MOUSE PROTEIN COUPLED	WT. OF PROTEIN (mg)	DRY WT. OF CnBr SEPHAROSE 4B (g)	CONDITIONS OF COUPLING	REMARKS
IgG	60	7	2 hr at 22°C	Still efficient after 2 yr
Fab	70	12	2 hr at 22°C	Still efficient after 2 yr
IgA	1	1	2 hr at 22°C and overnight at 4°C	Used to absorb anti-IgM. However the unabsorbed anti-IgM did not actually show any anti-IgA activity on gel diffusion analysis.
IgM	3	¾	2 hr at 22°C	Efficient for first run but subsequently very inefficient.
IgG_1	2·5	¾	2 hr at 22°C	Still efficient after 6 months.
IgG_{2a}	10	1½	2 hr at 22°C	Still efficient after 6 months.
Ascending part of 19S peak (free of IgA but containing IgM)	approx. 5	3	2 hr at 22°C and overnight at 4°C	Did not absorb out anti-IgM activity very efficiently.
Descending part of 19S peak of serum from germ free mice (containing IgM but no IgA)	1·6	3	Leave CnBr sepharose 4B in coupling buffer for 3 hr at 4°C before adding protein. Leave overnight at 4°C.	Did not absorb anti-IgM activity.

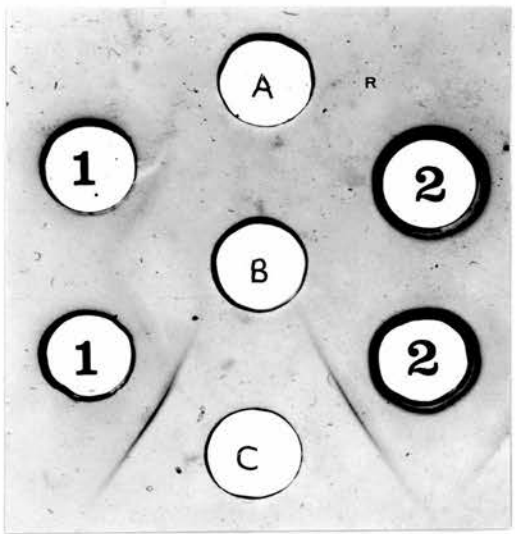
TABLE 9 IMMUNOABSORPTIONS NECESSARY BEFORE ANTISERA WERE SPECIFIC

ANTISERUM	IMMUNOABSORPTIONS
Anti-IgM	IgG
Anti-IgA	IgG, IgM, 19S fraction with no IgA
Anti-IgG_1	IgG_{2a}, Fab(1)
Anti-IgG_{2a} (I)	Fab(1), IgG_1, IgM
Anti-IgG_{2a} (II)	Fab(1)

1) Fab fragments were pooled from papain digestions of (a) mouse myeloma proteins; (b) IgG from normal serum.

Fig. 5 EFFECTIVENESS OF IMMUNOABSORBENT COLUMNS (1)

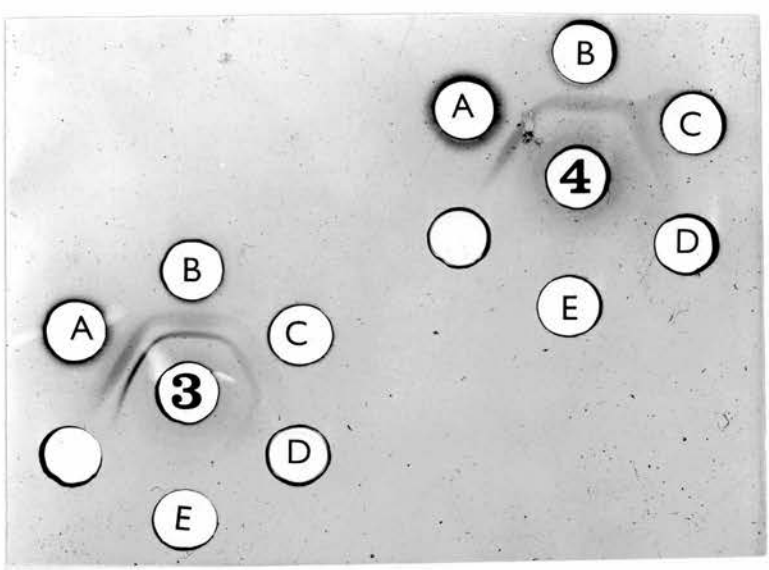
Mouse Fab immunoabsorbent column



A - mouse IgG_1
B - mouse Fab
C - mouse IgG_{2a}

1 - RAM IgG_{2a} (II) before absorption with Fab
2 - RAM IgG_{2a} (II) after absorption with Fab

Mouse IgM immunoabsorbent column

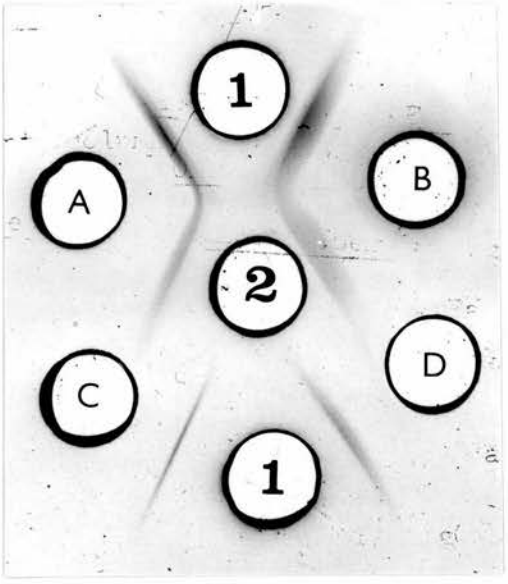


A - normal mouse serum
B - " " " ½
C - " " " ¼
D - " " " ⅛
E - " " " 1/16

3 - RAM IgA before absorption with IgM
4 - RAM IgA after absorption with IgM.

Fig. 6 EFFECTIVENESS OF IMMUNOABSORBENT COLUMNS (2)

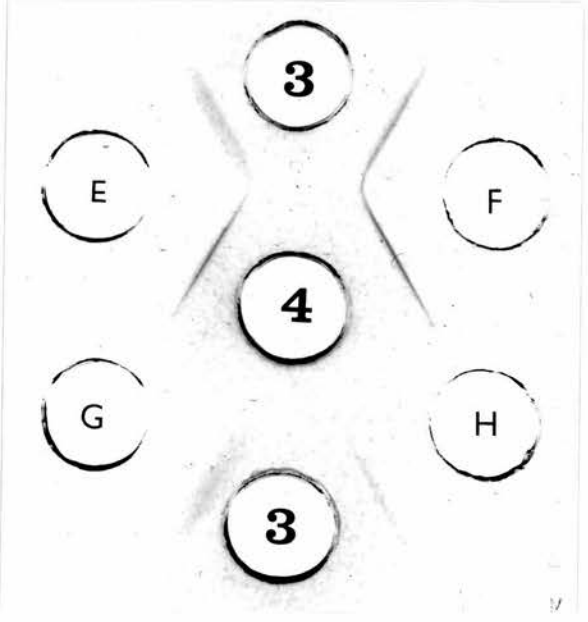
Mouse IgG_1 immunoabsorbent column



A - normal mouse serum
B - normal mouse serum 1/5
C - IgG_1 0·5 mg/ml
D - IgG_1 0·1 mg/ml

1 - RAM IgG_{2a} before absorption with IgG_1
2 - RAM IgG_{2a} after absorption with IgG_1

Mouse IgG_{2a} immunoabsorbent column

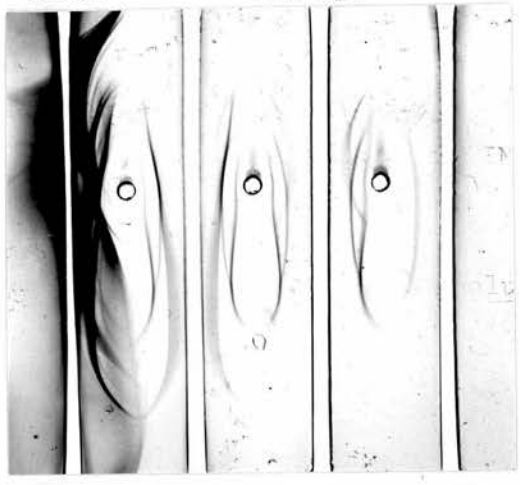


E - normal mouse serum
F - normal mouse serum 1/5
G - IgG_{2a} 0·5 mg/ml
H - IgG_{2a} 0·05 mg/ml

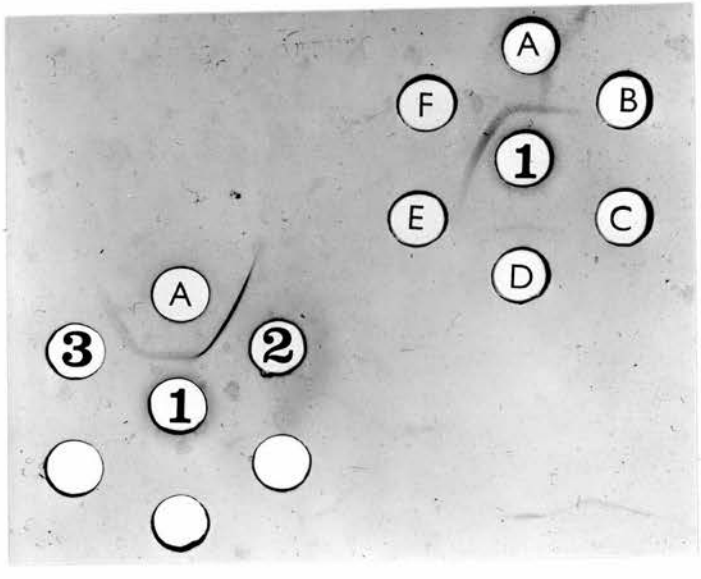
3 - RAM IgG_1 before absorption with IgG_{2a}
4 - RAM IgG_1 after absorption with IgG_{2a}

Fig. 7

EXAMINATION OF SPECIFICITY OF RABBIT ANTI-MOUSE (RAM) IgA



Wells contain normal mouse serum



A - normal mouse serum (NMS)
B - mouse IgG_1
C - mouse IgG_{2a}
D - mouse IgM
E - mouse Fab
F - mouse IgA

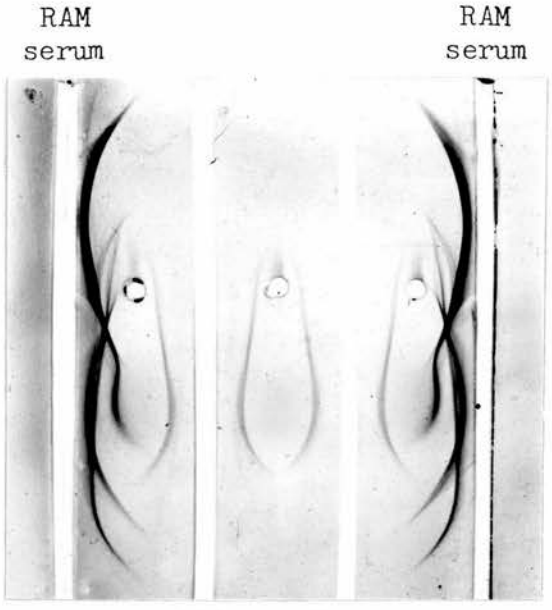
1 - RAM IgA
2 - RAM IgA (Meloy Laboratories)
3 - RAM IgA (Nordic Diagnostic)

Note

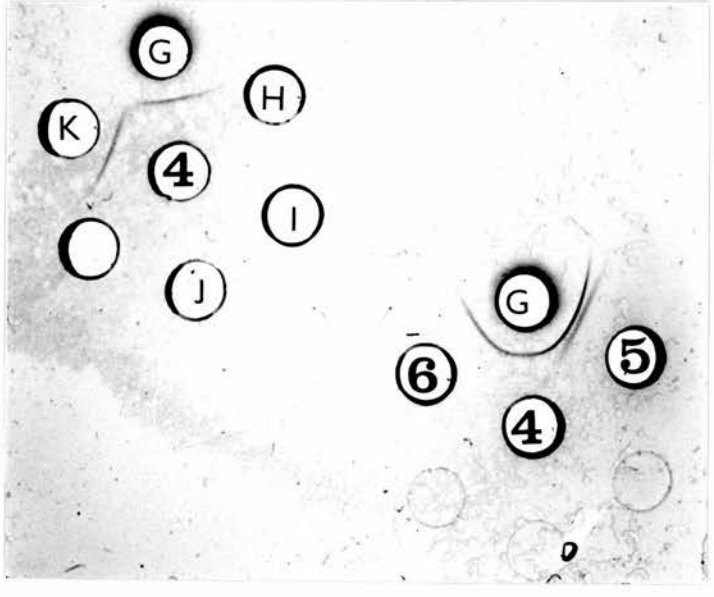
a) Single line on electrophoresis against NMS
b) Line of identity with homologous commercial antisera
c) Reaction only with IgA.

Fig. 8

EXAMINATION OF SPECIFICITY OF RABBIT ANTI-MOUSE (RAM) IgM



Wells contain MOPC 104E myeloma serum



G - normal mouse serum (NMS)
H - mouse IgG
I - mouse IgA
J - mouse Fab
K - mouse IgM

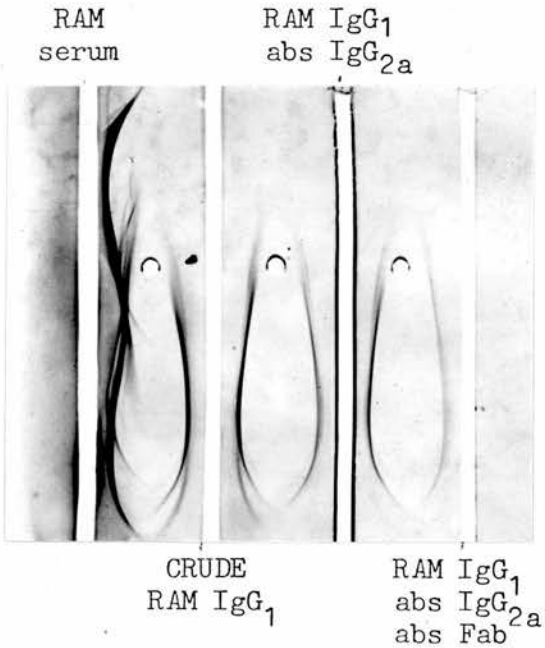
4 - RAM IgM
5 - RAM IgM (Meloy Laboratories)
6 - RAM IgM (Nordic Diagnostics)

Note

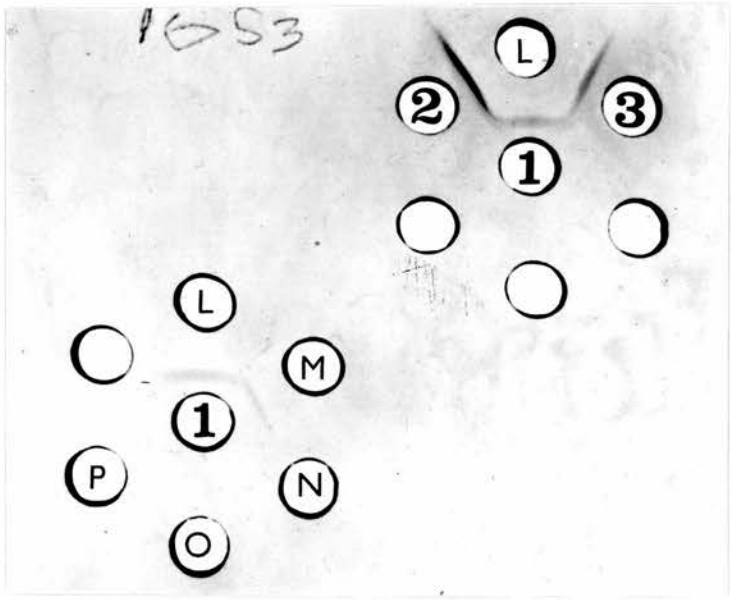
a) Single line on immunoelectrophoresis against NMS
b) Line of identity with homologous commercial antisera
c) Reaction only with IgM

Fig. 9

EXAMINATION OF SPECIFICITY OF RABBIT ANTI-MOUSE (RAM) IgG_1



Wells contain normal mouse serum



L - normal mouse serum (NMS)
M - mouse IgG_1
N - mouse IgG_{2a}
O - mouse Fab
P - mouse IgA + IgM

1 - RAM IgG_1
2 - RAM IgG_1 (Meloy Laboratories)
3 - RAM IgG_1 (Nordic Diagnostics)

Note

a) Single line on immunoelectrophoresis against NMS
b) Line of identity with homologous commercial antisera
c) Reaction only with IgG_1

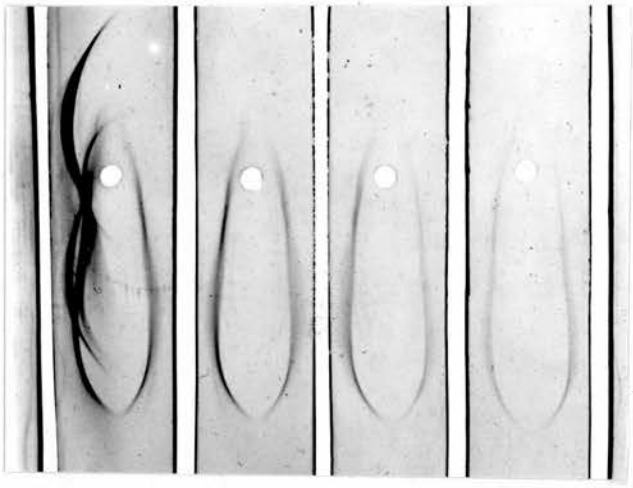
Fig. 10

EXAMINATION OF SPECIFICITY OF RABBIT ANTI-MOUSE (RAM) IgG_{2a} (I)

RAM serum

RAM IgG_{2a} abs Fab

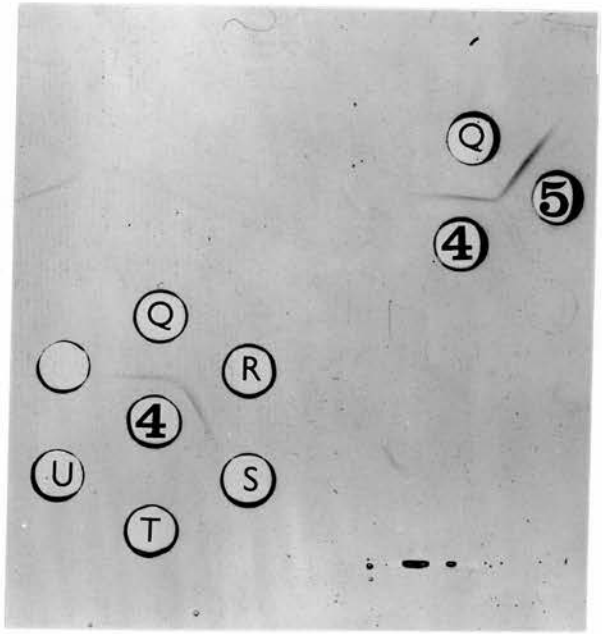
RAM IgG_{2a} abs Fab abs IgG_1 abs IgM



Wells contain normal mouse serum

CRUDE RAM IgG_{2a}

RAM IgG_{2a} abs Fab abs IgG_1



Q - normal mouse serum (NMS)
R - mouse IgG_{2a}
S - mouse IgG_1
T - mouse IgA + IgM
U - mouse Fab

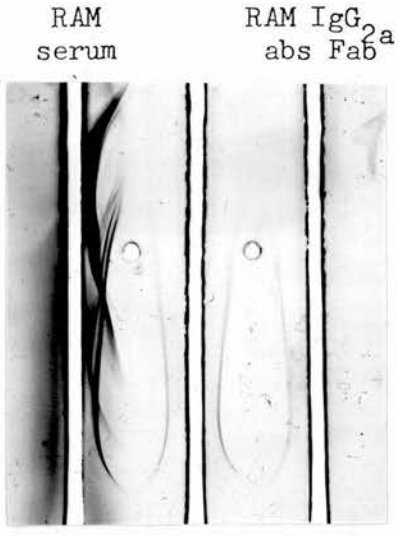
4 - RAM IgG_{2a} (I)
5 - RAM IgG_{2a} (Meloy Laboratories, Springfield, Va 22151)

Note

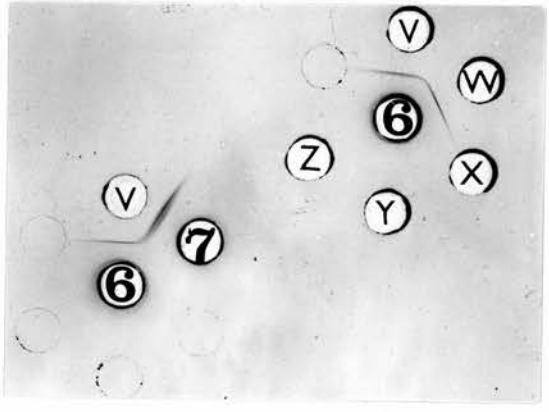
a) single line on immunoelectrophoresis against NMS
b) line of identity with homologous commercial antiserum
c) reaction only with mouse IgG_{2a}

Fig. 11

EXAMINATION OF SPECIFICITY OF RABBIT ANTI-MOUSE (RAM) IgG_{2a} (II)



Wells contain normal mouse serum



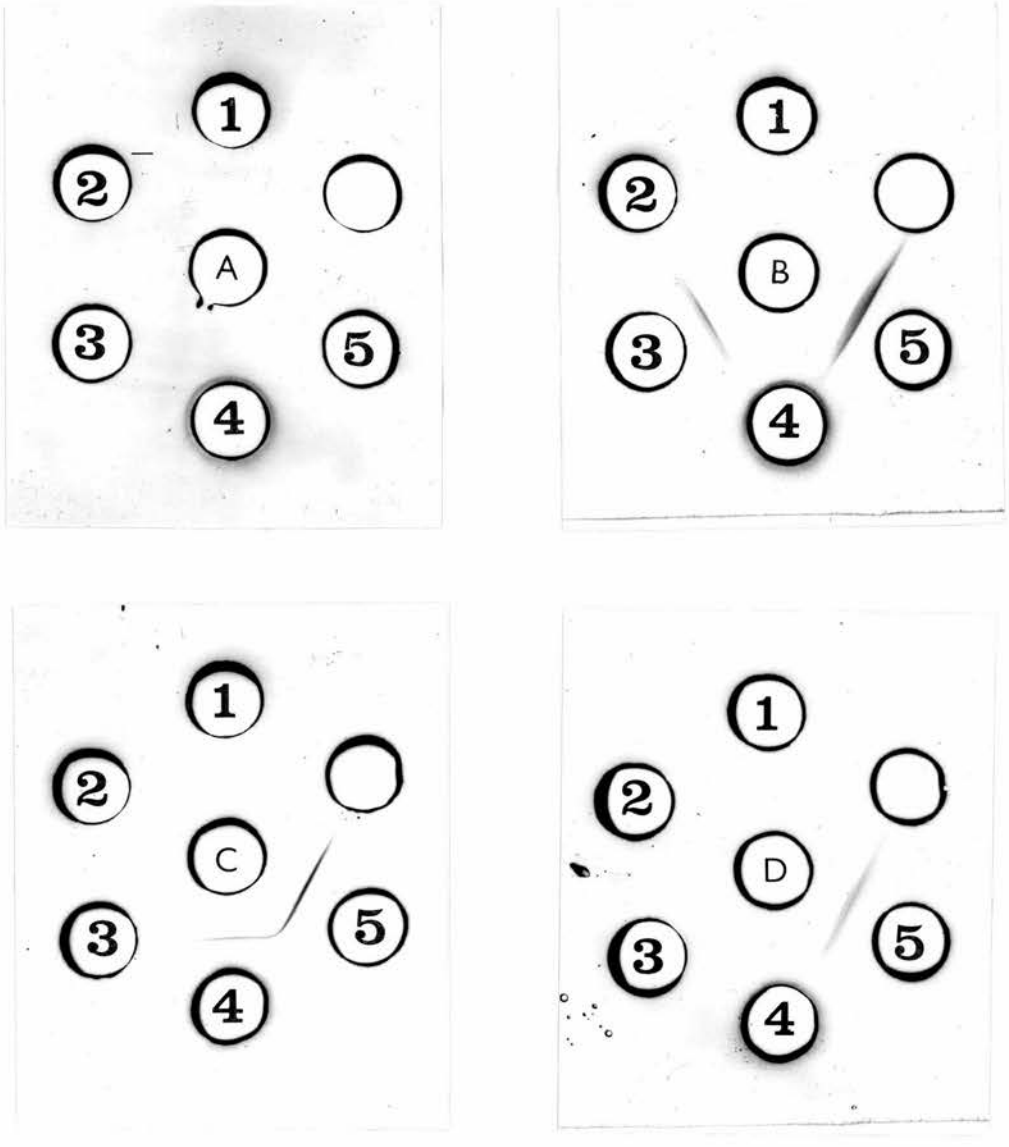
V - normal mouse serum
W - mouse IgG_{2a}
X - mouse IgG_1
Y - mouse IgA + IgM
Z - mouse Fab

6 - RAM IgG_{2a} (II)
7 - RAM IgG_{2a} (Meloy Laboratories)

Note

a) single line on immunoelectrophoresis against NMS
b) line of identity with homologous commercial antiserum
c) reaction only with IgG_{2a}

Fig. 12 EXAMINATION OF SPECIFICITY OF RABBIT ANTISERA TO MOUSE IMMUNOGLOBULINS, USING COMMERCIAL STANDARDS



Commercial Standards : A - mouse IgA; B - mouse IgG_1;
C - mouse IgG_{2a}; D - mouse IgG_{2b}.

Antisera : 1 - RAM IgA; 2 - RAM IgM; 3 - RAM IgG_1;
4 - RAM IgG_{2a}; 5 - RAM IgG.

Note
1) Commercial standards at 1 mg/ml (Litton Bionetics, Kensington, Md 20795)
2) Antisera only react with the appropriate antigen.

the following points may obviate the difficulties that did occur. They are, however, not the result of rigorous experimentation but of retrospective analysis, hindsight and generalisation.

i) A minimum of 1 mg per injection (more if the Fc fragment is injected) is necessary to raise an antiserum that can be used in Mancini analysis.

ii) I.m. is superior to s.c. injection.

iii) It is not necessary to inject the Fc fragment. It is preferable to inject the whole molecule and absorb the antiserum with Fab.

iv) In the production of anti-IgA ensure the injected antigen is free of IgM. This caused considerable difficulty because mouse IgM is difficult to separate from IgA and, furthermore, it is labile when coupled to Sepharose 4B. This is to be compared with IgG coupled to Sepharose 4B which is still active after 2 years.

3) CELL LINES

a) Origin

Fibrosarcomas were induced in 8 - 10 week old CBA and A/HeJ mice by an intramuscular injection of methylcholanthrene (0·5 mg) in trioctanoin (0·1 ml). CBA embryonic fibroblasts were obtained from minced embryos, usually in the third week of gestation. Normal fibroblasts were obtained from the kidneys of adult mice. Plasmacytomas were originally produced in BALB/c mice by the injection of adjuvant or mineral oil; they were generously donated to this laboratory by those listed in Table 1.

b) In vivo propagation of tumours

The in vivo propagation of plasmacytomas and fibrosarcomas was carried out by an s.c. injection of a cell suspension. A plasmacytoma cell suspension was made by mincing up tumour tissue with a Teflon homogeniser, decanting the cell suspension and injecting measured volumes. This crude technique was adequate for routine propagation of plasmacytomas, but for experiments with the CBA fibrosarcoma the following aseptic technique was used. Tumour tissue was cut into small pieces and poured into a Melnick flask. The supernatant was decanted, more sterile Dulbecco A + B added and the supernatant again discarded. After a further wash in pronase/Dulbecco (2·5 g pronase, 20 mg DNAse per litre of Dulbecco A + B) at 37°C for 30 minutes, the tumour tissue was resuspended in pronase/Dulbecco and slowly stirred for 10 minutes at 37°C. The supernatant was decanted through 2 ply gauze into a flask containing Dulbecco A + B at 4°C and the tissue remaining in the flask further digested as above. Cells obtained in this way were washed three times in Dulbecco A + B before being used. They were at least 90% viable.

c) In vitro propagation of tumour cell lines

A cell suspension was made as in the previous section and 4×10^6 cells in 10 ml growth medium (RPMI - 1640, 10% foetal calf serum, 2 mM/L glutamine, 100 units crystamycin/ml) seeded in tissue culture flasks kept at 37°C. The next day the nonadherent cells were reseeded (1×10^6 cells in 10 ml) and at intervals of 3 - 4 days were sub-cultured at this density.

d) In vitro propagation of embryo cell lines

A CBA mouse in the third week of gestation was killed by ether inhalation and the uterus, with the embryos, removed aseptically. After detaching the embryos from the uterus they were decapitated, their viscera discarded, then the remains cut into small pieces and immersed in trypsin/Dulbecco (2·5 g trypsin, 20 mg DNAse per L. of Dulbecco solution) in a Melnick flask. The subsequent procedure was as for the digestion of tumour cells, except that the period of stirring was 20 minutes instead of 10 minutes.

Embryonic fibroblasts were propagated in vitro by the same method as for tumour cells. It was observed that whereas tumour cells had a seemingly limitless existence, the life span of the embryo lines was about 5 sub-cultures.

e) In vitro propagation of normal kidney fibroblasts

This method was based on that of Paul (171). Briefly an adult mouse kidney, minus the membrane, was minced and left in trypsin /Dulbecco (2·5 g trypsin, 20 mg DNAse per L. of Dulbecco A + B) for 20 minutes at room temperature. The supernatant was spun down and discarded then the minced kidney was resuspended in 20 ml of trypsin/ Dulbecco and kept at 4°C overnight. Next day the cells were maintained at 37°C for 30 minutes in a water bath, large pieces of tissue allowed to settle and the cell suspension removed and washed in cold Dulbecco A + B. Undigested tissue was re-incubated in trypsin/ Dulbecco for a further 30 minutes at 37°C, and the supernatant containing cells removed.

Cells were washed three times in Dulbecco A + B before seeding

in tissue culture vessels at 5×10^6 cells per bottle. The *in vitro* propagation was similar to the propagation of embryo cell lines. Again it was found that non-malignant mouse fibroblasts had a restricted life-span under culture conditions.

f) **Storage of cells under liquid nitrogen**

Freshly excised tumour tissue was finely minced and left for 1 hour in the "freezing solution" (199 medium, 20% foetal calf serum, 100 units/ml Crystamycin, 7·5% dimethylsulphoxide). Aliquots were poured into suitable containers and cooled at 1°C per minute over the range 0°C to -15°C. After freezing down to about -30°C the ampules were stored in the vapour phase of liquid nitrogen. Alternatively a cell suspension could be frozen down in a similar manner.

g) **Tumour transplantation and measurement**

s.c. transplantation The CBA fibrosarcoma was transplanted by a s.c. injection into the right thigh of 10^4 or 10^5 pronase-digested tumour cells (see Materials and Methods 3b) or *in vitro* cultured tumour cells (see Materials and Methods 3c). Prior to these studies the CBA and A/HeJ fibrosarcomas had been transplanted 18 and 23 times respectively. Tumour growth was assessed throughout the period of experimentation by measuring two diameters at right angles. It could be readily demonstrated that the CBA fibrosarcoma was immunogenic in the syngeneic host (Table 37).

i.v. transplantation A putatively more sensitive technique than measurement of tumour diameters is the enumeration of tumour cell colonies in the lungs after i.v. injection of tumour cells.

This method was used to assess the effect of serum from various sources on the ability of tumour cells to form lung colonies. The serum (diluted 1/5) was incubated with CBA fibrosarcoma cells (2×10^6/ml) for 45 minutes at 37°C. After washing once mice were injected i.v. via the tail vein with 5×10^4 cells. Fourteen days later they were killed by cervical dislocation, their lungs excised then washed and immersed in fixative. The number of colonies on both sides of each lobe was counted using a low power magnifying glass. The Bouin's solution used as fixative consisted of saturated picric acid solution (75 ml), 40% formaldehyde (25 ml), glacial acetic acid (5 ml).

4) ASSAY TECHNIQUES

a) Antiglobulin assay

An antiglobulin assay was used to detect mouse immunoglobulin binding to tumour target cells *in vitro*. The specificity of binding was examined by a) absorption with non-malignant cells; b) using non-malignant cells as targets in the antiglobulin assay. This technique needed: target cells; test serum in which antibodies to the target cells were suspected; and a reagent, labelled with radioisotope, capable of reacting specifically with immunoglobulin in the test serum. Thus the γ-radiation retained by the cells was proportional to the anti-target cell antibody in the test serum.

Reagents for use in the antiglobulin assay were prepared as follows. Mouse IgG was prepared by fractionating whole serum on G-200 sephadex, then precipitating the 7S fraction with 16% Na_2SO_4.

The precipitate was analysed as pure mouse IgG by gel diffusion and immunoelectrophoresis. Rabbit anti-mouse IgG was prepared by injecting a New Zealand white rabbit s.c. with approximately 1·5 mg of mouse IgG in Freund's complete adjuvant, and repeating the dose two weeks later. After another two weeks a final i.p. injection of approximately 1·5 mg mouse IgG in Alhydrogel was given, then after a further two weeks the rabbit was bled from the ear. As judged by gel diffusion precipitin analysis this antiserum reacted with all the principal immunoglobulin classes and sub-classes in mouse serum (Fig. 13). Before use in the antiglobulin assay the antiserum, or normal rabbit serum used as control, was absorbed with 10^7 cultured tumour cells per millilitre of serum, for one hour at 22°C.

Rabbit IgG was prepared by batch chromatography on DEAE cellulose as described by James (172). Goat anti-rabbit IgG was prepared by a s.c. injection of 8 mg of rabbit IgG in Freund's complete adjuvant, followed by a similar injection six weeks later. After a further eight days the goat was bled. It should be noted that the goat was also bled prior to immunisation to provide a normal goat serum control.

The IgG from both the goat anti-rabbit IgG serum and the pre-bleed sample were prepared by batch chromatography on DEAE cellulose. Both goat IgG preparations were labelled with ^{125}I by the iodine monochloride method (see Materials and Methods 5).

In these studies four variations of the basic technique have been used (Table 10). The methods for each are as follows:-

A. CBA fibrosarcoma cells (approx. 2 x 10^6) were incubated at 4°C for 30 minutes with the test mouse serum (1 ml diluted 1/12) in suitable test tubes, then spun down and washed twice. The cells were then resuspended in rabbit anti-mouse IgG serum (1 ml diluted 1/20 - 1/80), incubated at 4°C for 30 minutes and again spun down and washed twice. Finally the cells were incubated with ^{125}I-labelled globulin fraction of goat anti-rabbit IgG (1 ml 60 µg - 300 µg) and processed as before. After the final wash the cells were counted in a γ-scintillation spectrometer.

In this method all dilutions and washings used Eagles minimal essential medium plus 5% foetal calf serum, buffered with 20 mM hepes (BDH Chemicals Ltd., Poole), and preserved with 100 units/ml of Crystamycin (Glaxo Laboratories Ltd., Greenford, England).

Preliminary experiments incorporated a full set of controls including normal rabbit serum and ^{125}I-globulin fraction of normal goat serum. It was soon evident that non-specific absorption due to these reagents was minimal, so their routine use was discontinued.

Using this technique the immunoglobulin in the test serum binding to the target cells *in vitro* was expressed as the Absorption Ratio (A.R.)

$$AR = \frac{\text{COUNTS PER UNIT TIME IN TUBES WITH TEST SERUM}}{\text{COUNTS PER UNIT TIME IN TUBES WITH NORMAL SERUM}}$$

B. The target cells (2 - 4 x 10^4), cultured *in vitro* for 8 - 50 days, were seeded into each well of a disposable plastic microculture plate (Linbro 15-FB-96 or Nunclon A/S Nunc, Denmark),

covered with a lid and incubated overnight at 37°C in a humid atmosphere to allow the cells to adhere. The cells were washed once with medium (200µl) then mouse serum from the various experimental groups (25 - 100 µl diluted 1/5 - 1/30) was added, and the plate incubated at 37°C for 30 minutes. The plate was cooled to 15° -20°C and the cells washed three times with medium (200 µl each). Rabbit anti-mouse IgG (50 - 100 µl diluted 1/50 - 1/100) was then added, the plate incubated at 15 - 20°C for 30 minutes and the cells again washed three times (200 µl each). Finally ^{125}I-labelled immunoglobulin from goat anti-rabbit IgG (5 - 10 µg) was added, the plate incubated for 30 minutes at 15 - 20°C and the cells washed as before. After each wash the cells were spun down at 300 rpm for 3 minutes, and when dry after the final wash the plate was sprayed with plastic wound dressing, individual wells cut out with a hot wire, and the remaining δ-radiation counted in a scintillation spectrometer.

In this method the washing of cells and dilution of reagents was in RPMI 1640 supplemented with 10% foetal calf serum, buffered with 20 mM Hepes, and preserved with 100 units per ml of penicillin/streptomycin.

Using this technique the immunoglobulin in the test serum binding to the target cells <u>in vitro</u> was expressed as the Absorption Ratio as in Method A. Alternatively, individual sera in an experimental group were examined and the results (counts per unit time) expressed in scattergram form.

C. In this method a number of modifications were introduced

which were intended to make the assay more sensitive. Thus the cells were removed from the reaction tubes prior to γ-counting to reduce the background γ-counts, and a specific rabbit antibody to mouse IgG (eluted from a mouse IgG immunoabsorbent column) was used as the antiglobulin reagent.

CBA fibrosarcoma cells ($4 - 7 \times 10^5$) were incubated at 37°C for 30 minutes with the test mouse serum (0·2 ml diluted 1/7 - 1/11) in suitable test tubes, then spun down and washed three times. (1 ml each) The cells were then resuspended in the antiglobulin reagent (0·2 ml. For dilution see Results) and incubated at 15 - 20°C for 30 minutes. They were then washed four times (1 ml each) before being transferred to fresh tubes, where they were washed once more before being counted in a γ-scintillation spectrometer.

In this method the washing of cells and dilution of reagents was in RPMI 1640 plus 10% foetal calf serum as in Method B.

The immunoglobulin in the test serum binding to the target cells in vitro was expressed as the Absorption Ratio (AR),

$$AR = \frac{\text{COUNTS PER UNIT TIME WITH CELLS INCUBATED WITH TEST SERUM}}{\text{COUNTS PER UNIT TIME WITH CELLS INCUBATED WITH NORMAL SERUM}}$$

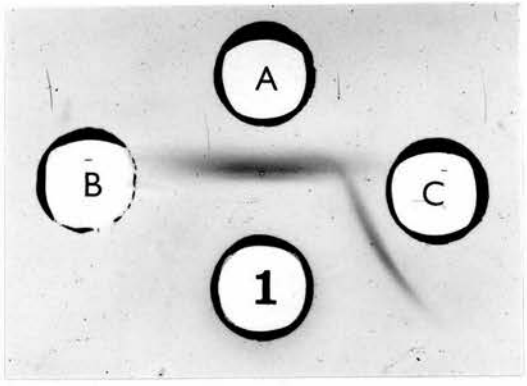
D. This method is the same as Method B except that: a) the cells were plated out the same day as the assay; b) the cells were transferred to a fresh plate for γ-scintillation counting as follows. Ice cold Dulbecco A (100 µl) was used for the last wash to remove foetal calf serum. After spinning down, the supernatant was gently removed by aspiration, replaced by fresh ice cold Dulbecco A (100 µl),

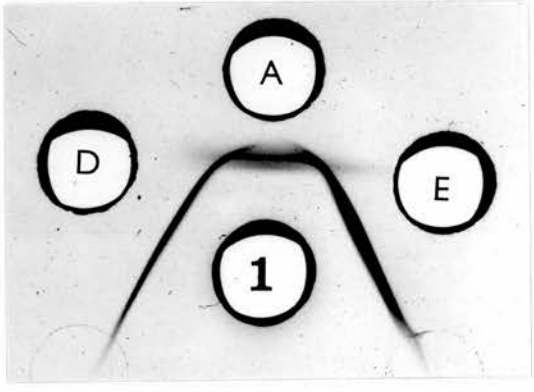
TABLE 10 VARIATIONS OF ANTIGLOBULIN ASSAY (CHRONOLOGICAL ORDER)

	TARGET CELLS	ANTIGLOBULIN REAGENT(S)	PROCESSING OF CELLS BEFORE γ-SCINTILLATION COUNTING	COMMENTS
A	Single cell suspension of *in vitro* cultured or pronase digested cells.	RAM IgG serum, followed by the globulin fraction of GAR IgG, labelled with ^{125}I.	None - cells counted in γ-counter in reaction tubes.	Not sensitive - limited in number of samples processed due to separate washing and centrifugation of each sample.
B	*In vitro* cultured cells plated out the day before use into each well of a microculture plate.	As above	None - cells counted in γ-counter in separate plastic wells of microculture plate.	More sensitive than A. Economical in cells and serum. Faster than A.
C	Single cell suspension of *in vitro* cultured cells.	Globulin fraction of GAR IgG, labelled with ^{125}I - made specific for mouse IgG by elution from solid phase immunoabsorbent.	Removed from reaction tubes and γ-counted in fresh tubes.	About same sensitivity as B.
D	Single cell suspension of *in vitro* cultured cells dispensed into each well of a microculture plate on day of assay.	RAM IgG serum, followed by the globulin fraction of GAR IgG, labelled with ^{125}I.	Removed from plastic wells and γ-counted in fresh plastic wells.	More sensitive than B.

Abbreviations - RAM - Rabbit anti-mouse
GAR - Goat anti-rabbit

Fig. 13 SPECIFICITY OF RABBIT ANTI-MOUSE IgG USED IN THE ANTIGLOBULIN ASSAY





A - mouse IgG
B - mouse IgA
C - mouse IgM
D - mouse IgG_1
E - mouse IgG_{2a}

1 - RAM IgG

Note

RAM IgG reacts with all classes and sub-classes of mouse immunoglobulin tested, except IgA.

the cells gently aspirated and stirred using a 100 µl Oxford pipette, then transferred to the fresh plate kept on ice. Transfer was completed by adding 100 µl of trypsin/sodium versinate solution PH8 (0·025% / 0·02%), the cells being transferred as before.

In this method the washing of cells and dilution of reagents was in RPMI 1640 plus 10% foetal calf serum as in Method B.

On two occasions (when using antisera to mouse immunoglobulin subclasses, and when finding the optimum conditions for such an experiment) goat anti-mouse immunoglobulin serum was used together with ^{125}I-labelled globulin fraction of rabbit anti-goat IgG. This was because only goat antiserum to mouse sub-classes was available.

b) Assay for cytostatic antibody

This assay is basically as described by Le Mevel and Wells (155). Target cells (5×10^3 - 1×10^4), cultured as described in Materials and Methods 3 c, were plated into each well of a plastic microculture plate (Limbro 15-FB-96 or Nunclon A/C Nunc, Denmark) and incubated at 37°C overnight. The medium was gently aspirated, the cells washed in RPMI growth medium (Materials and Methods 3c) (200 µl), and test serum (25 µl) at a range of dilutions added to each well. The plate was incubated at 37°C for 30 minutes, then guinea pig complement (25 µl diluted 1/12) added and the plate reincubated at 37°C for 6 hours. After aspirating the serum and complement, ^{125}I-labelled iododeoxyuridine (0·5 µ Ci in 0·1 ml medium) was added and the plate reincubated for 18 hours. Finally the cells were washed 3 times in medium (200 µl), air dried, sprayed with plastic wound dressing, and the plastic wells separated on a hot wire and

and counted in a γ-scintillation spectrometer.

The percentage cytostasis was expressed as

$$\left[1 - \frac{\text{CPM with test serum and complement}}{\text{CPM with control serum and complement}}\right] \times 100$$

or if control serum showed cytostatic activity

$$\left[1 - \frac{\text{CPM with serum and complement}}{\text{CPM with complement}}\right] \times 100$$

c) Titration of anti-C.parvum antibody by latex agglutination

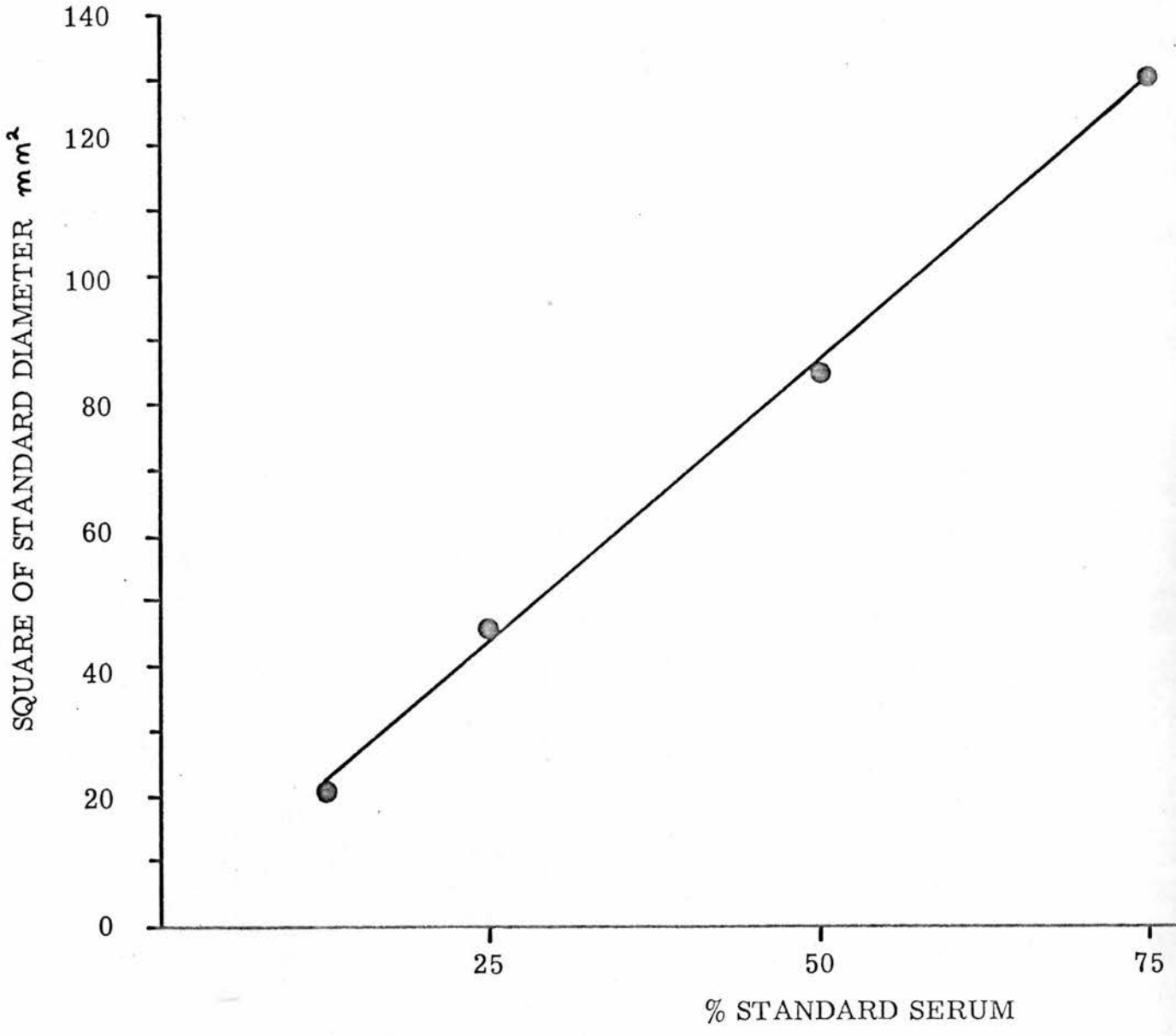
The bacterial and latex (Difco; 0·81 µm) suspensions were first adjusted to give optical densities (540 nm) of 38 and 192 respectively. From these stock suspensions (defined as 100%) a mixture containing 5% bacteria and 0·5% latex (v/v) was prepared in glycine buffer pH 8·2, containing 0·1% BSA (GBSA). The buffer was added last after giving the bacteria a few minutes to coat the latex particles. The test sera were diluted (25 µl volumes) in GBSA and one volume was dispensed into each well of a plastic microtitre tray, followed by one volume of the bacteria-latex suspension. The plates were incubated at 37°C for 2 hours, spun at 500 g for 20 minutes and read directly for agglutination over a direct light source. Results are expressed as the $\log_2$ reciprocal of the antiserum end point dilution (173).

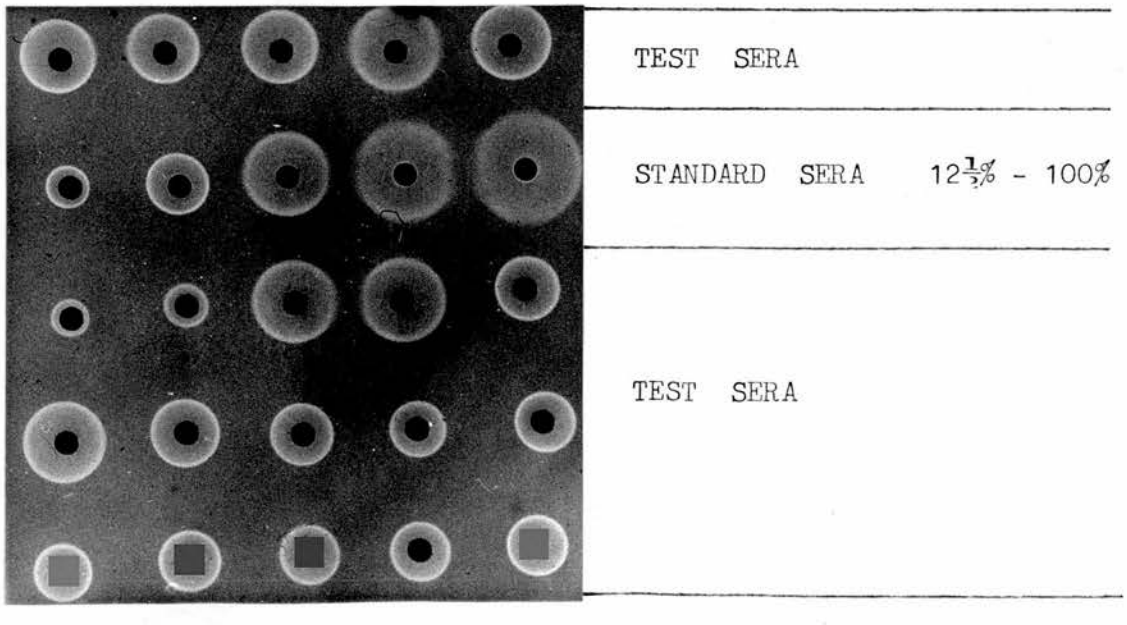
d) Estimation of immunoglobulin class and sub-class levels in mouse serum

The antisera for these estimations were either purchased from

Fig. 14

CALIBRATION CURVE FOR ESTIMATION OF IgG_{2a} IN MOUSE SERUM





Meloy Laboratories (Springfield, Va 22151) or prepared in this laboratory by the methods outlined in Materials and Methods 2.

Estimations of the immunoglobulin sub-class content of individual sera were performed by the single radial immunodiffusion method of Mancini et al. (174). Duplicate assays were undertaken on each serum and all plates contained a range of dilutions of a standard serum. The standard serum was a pool from 30 male and 30 female CBA mice aged 12 weeks. In early experiments the immunoglobulin levels of test sera were expressed as a percentage of this standard serum; later results have been expressed as absolute values.

A calibration curve, using 12½, 25, 50, 75 and 100% standard mouse serum, was drawn for each plate. The test mouse serum, diluted by a factor of say 3, was read off the calibration curve and multiplied by the dilution factor. Examples of this procedure are shown in Fig. 14.

In later experiments serum immunoglobulin levels were expressed as absolute values. To do this the standard mouse serum was titrated against purified mouse immunoglobulin class and sub-class myeloma proteins of known concentration (Litton Bionetics Inc., Kensington, Md, USA). It was found to contain 31 mg IgM/dl, 20 mg IgA/dl, 66 mg IgG_1/dl, 380mg IgG_{2a}/dl, 7hmg IgG_{2b}/dl.

e) K-cell cytotoxicity assay

This assay gives a measure of the ability of cells to participate in antibody-dependent lymphocyte cytotoxicity. In these experiments effector cells were mouse spleen cells depleted of macrophages by glass adherence; target cells were ^{51}Cr-labelled chicken

red blood cells; and antibody to target cells was rabbit anti-chicken red blood cell. Details of this method are given in Calder et al. (175). K-cell cytotoxic activity was expressed as:-

$$\frac{\% \text{ release with antibody and spleen cells} - \% \text{ release with spleen cells alone}}{\% \text{ maximum release with antibody plus complement} - \% \text{ release with spleen cells alone}}$$

f) Plaque-forming cell assay for antibody to SRBC

This technique was based on that originally described by Jerne et al. (176), extended to measure anti-SRBC antibody of all classes and sub-classes. The assay measured the number of cells secreting antibody to SRBC, the 19S antibody being detected by its ability to fix complement directly and cause lysis of the surrounding SRBC - the so-called direct plaques. Detection of 7S antibody to SRBC required the use of an antibody to immunoglobulin secreted by the antibody forming cells before plaques would form on the addition of complement - the indirect plaques.

The monolayer adaption of the plaque-forming cell assay was used in these studies, details of which are given elsewhere (177).

g) Assay for antibody response to alum-BSA

This technique is based on that originally described by Farr (178). It measures the antibody specific for a defined antigen in terms of Antigen Binding Capacity and Releative Binding Affinity. Briefly, the control and test sera were incubated with excess BSA labelled with ^{125}I as described in Materials and Methods 5, and the complexed separated from free antigen by precipitation with 50%

ammonium sulphate. After washing, the precipitate was counted in a γ-counter, thereby obtaining a measure of ^{125}I-BSA in complex form.

The results were used, together with a standard computer programme, to give values of Antibody Binding Capacity (which gives a measure of the total amount of antibody in an antiserum, of all immunoglobulin classes, that is available to combine with antigen) and Relative Binding Affinity (which gives a measure of the binding strength between antibody and antigen).

5) USE OF ISOTOPES

The following low energy γ-emitters were used: ^{51}Cr in the form of sodium chromate as a cell label; ^{125}I in the form of iododeoxyuridine as a thymidine analogue; and ^{125}I in the form of sodium iodide for protein labelling. All were obtained from the Radiochemical Centre, Amersham. Standard safety precautions were taken when using the isotopes, although these particular radiochemicals are relatively innocuous due to their low energy emissions and short half-lives.

The (^{51}Cr)-sodium chromate and (^{125}I)-iododeoxyuridine were used in the above form: (^{125}I) -sodium iodide was used to label immunoglobulin by the method of Hunter and Greenwood (179). Briefly the protein (5 - 20 mg) in 2 ml of physiological saline was added to the isotope (5m Ci) with continuous stirring. This was followed by the immediate addition of 0·2 ml of chloramine-T (0·5 mg/ml). After stirring for 5 minutes the reaction mixture and washings were transferred to a 8/32" Visking Cellulose dialysis sac. The final volume of approximately 4 ml was dialysed for 3 days against four

changes of physiological saline at 4°C, the first dialysis buffer containing 0·1% KI.

After an experiment the γ-radiation was counted in an LKB Wallac 1280 Ultragamma: in all cases the background counts (approx. 120 CPM) were an insignificant percentage of the sample counts.

6) PRESENTATION OF RESULTS

The tumour diameters for each group were expressed as geometric means together with the limits of one standard error from the mean; anti-C.parvum antibody titres were expressed as the arithmetic means of the $\log_2$ titre $\pm$ one standard error; and immunoglobulin levels were expressed as the arithmetic mean values $\pm$ one standard error. Antibody, detected by the antiglobulin technique, was expressed as counts per unit time in scattergram form when individual mouse sera were tested, and as counts per unit time or Absorption Ratios when serum pools were tested. Each sample was tested in duplicate or triplicate and an experimental group or serum pool consisted of at least five mice.

The significance of the results was determined by the Student 't'-test procedure. Values of $P \leq 0{\cdot}05$ were regarded as significant.

7) ADJUVANTS USED

The adjuvants used in these studies were: C.parvum CN 6134 (Wellcome Research Laboratories, Beckenham, Kent); C.parvum 10387, Propionibacterium freudenreichii 10470 (Dr. W.H. McBride, Department of Bacteriology, University of Edinburgh); B.C.G. (Glaxo Laboratories, Greenford, Middlesex); Bordetella pertussis (Wellcome Reagents Ltd.,

Beckenham, Kent); complete and incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan).

The adjuvants obtained from commercial sources were standard preparations; the adjuvants grown in the Department of Bacteriology, Edinburgh, were formalin fixed.

A formalin fixed strain of C.parvum (CN 6134) was used in these studies, unless otherwise stated.

RESULTS

1. DEVELOPMENT OF ANTIGLOBULIN ASSAY

a) Variation of the uptake of mouse immunoglobulin with the age of the tumour target cells

The first antiglobulin technique used to detect the *in vitro* binding of mouse immunoglobulin to target cells was Method A (see Table 10). It was, however, soon found to be cumbersome and wasteful of time and reagents. It is illustrated here by an experiment designed to test whether freshly prepared pronase-digested, or *in vitro* cultured tumour target cells were preferable in the antiglobulin assay. The results (Table 11) are expressed as Absorption Ratios (AR).

It can be seen that there was no significant absorption of immunoglobulin from tumour bearing mouse serum onto cells of any age. However the anti-CBA fibrosarcoma serum appeared to be absorbed onto "younger" cells to a greater extent than onto "older" cells. This could be due to antigen deletion, but recent results from this laboratory (102) have shown that a freshly excised pronase-digested tumour cell suspension contains typically 30% Fc-receptor bearing cells, which decreases gradually to 0% on *in vitro* culture. Therefore, it may be that the assay is detecting uptake of immunoglobulin via the Fc-receptor of infiltrating host cells.

b) Sensitivity of antiglobulin Method A compared with Method B

Method A, as used in Results 1a, is limited in the number of individual samples that can be processed. Method B has the

advantages that numerous samples can be processed at once in a microculture plate, and it is economical in serum and cells. Using in vitro cultured CBA fibrosarcoma target cells the two methods were compared. It can be seen from Table 12 that Method B was more sensitive than Method A at the dilutions of reagents studied.

c) Optimum dilutions of reagents in antiglobulin Method B

Before starting any serious experimental work the conditions (dilutions of reagents etc.) were optimised to give the highest Absorption Ratios. In this series of experiments (see Tables 13, 14, 15) the positive serum used in Results 1 a and b was again used, and in addition a syngeneic serum which a priori would be expected to yield anti-tumour antibody. This serum was from CBA mice exsanguinated 14 days after their syngeneic tumour grafts had been excised. The target cells used in the antiglobulin assay were cultured CBA fibrosarcoma cells.

On the basis of the results in Tables 13, 14 and 15 it was decided to proceed using the following dilutions:-

Mouse serum : $^{1}/_{5}$

Rabbit anti-mouse IgG : $^{1}/_{120}$

^{125}I-goat anti-rabbit IgG : 5 - 10 µg per well

d) Examination of CBA fibrosarcoma target cell loss during antiglobulin Method B

A possible objection to this type of assay is that prolonged washing and spinning down of cells may lead to cell loss. To counter this objection the target cells were labelled with ^{51}Cr (1 µ Ci per well) for 1 hour at 22°C, then after washing twice a standard antiglobulin

assay was performed. The control in this experiment was cells labelled and washed as above but otherwise untouched until the end of the assay. Results are expressed as a percentage of this control and each sample was tested in quadruplicate.

The results (Table 16) show that the cells subjected to multiple washings and centrifugations of the assay (16 in all) did not lose more than 20% of their original number. The results were corrected for channel overlap of the γ-scintillation spectrometer as follows. 47% of the counts recorded in the ^{51}Cr channel for a ^{51}Cr sample appeared in the ^{125}I channel. Therefore the counts for the ^{125}I sample must be reduced by 47% of the ^{51}Cr counts in the ^{51}Cr channel. Fortunately the channel overlap from the ^{125}I to the ^{51}Cr channel was negligible (2·5%).

e) Absorption of reagents to plastic reaction wells in Method B

Another objection that can legitimately be advanced against the antiglobulin assay as used in Methods A and B is that absorption of reagents to the plastic wells might constitute an unacceptable proportion of the final γ-counts. To examine this the Absorption Ratio (AR) of serum from C.parvum-treated mice was plotted against the dilution of mouse serum in plastic wells with and without CBA fibrosarcoma target cells.

It was expected that the AR of serum from C.parvum-treated mice would approach unity in wells without target cells. At the dilutions studied this was true (Fig. 15). However, the counts in empty wells with normal serum were a significant proportion of the counts in wells with target cells plus normal serum (approximately 75%). Thus, in

antiglobulin Method B it is only valid to draw conclusions from the Absorption Ratios, ie. the relative uptake of the test serum against normal serum, and not from the absolute γ-counts.

This difficulty could be overcome by using Method C or D in which the cells were transferred from the tubes or wells used in the antiglobulin assay to fresh tubes or wells for γ-counting. Using Method C (Fig. 16) the Absorption Ratio of serum from C.parvum-treated mice was at least as high as in Method B. Antiglobulin Method D is illustrated in the next section.

f) Optimum dilutions of reagents in antiglobulin Method D

Antiglobulin Method D was used in two modifications: with rabbit anti-mouse IgG plus ^{125}I-labelled globulin fraction of goat anti-rabbit IgG; and with goat anti-mouse immunoglobulin plus ^{125}I-labelled globulin fraction of rabbit anti-goat IgG. This series of experiments examines whether variations in the concentration of mouse serum, or of the ^{125}I-labelled antiglobulin reagent, affected the result of the antiglobulin assay.

It can be seen from Table 17 that using the first variation the lowest concentration of mouse serum, combined with the highest concentration of ^{125}I-labelled antiglobulin reagent, gave optimum uptake of serum from the experimental groups (compared to normal serum). Conversely, using the second variation comparable results were only observed at the highest concentration of mouse serum and the lowest of ^{125}I-labelled antiglobulin reagent (Table 18). These conditions were used in subsequent experiments.

TABLE 11 VARIATION OF UPTAKE OF MOUSE IMMUNOGLOBULIN WITH THE AGE OF CBA FIBROSARCOMA TARGET CELLS

AGE OF TUMOUR CELLS (NUMBER OF SUBCULTURES)	$\frac{PS}{NMS}$ [a]	$\frac{TMS}{NMS}$ [a]
0	2·7	1·3
3	2·3	1·2
7	2·3	1·1
10	1·7	~1
17	1·7	1·1

a) NMS - normal serum from CBA mice

TMS - tumour bearer serum from CBA mice bearing a syngeneic fibrosarcoma.

PS - BALB/c anti-CBA tumour cell serum.

Note

There was no significant absorption by TMS onto cells of any age; however, the anti-CBA serum seemed to be absorbed onto "younger" cells more than to "older" cells. It might therefore be preferable not to use cells "older" than seven generations.

TABLE 12 COMPARISON OF ANTIGLOBULIN METHODS A AND B : VARIATION OF MOUSE SERUM DILUTIONS

DILUTION OF MOUSE SERUM	METHOD A		METHOD B	
	PS[a] / NMS	TMS[a] / NMS	PS / NMS	TMS / NMS
$^1/_5$	NT[b]	NT	3·5	1·20
$^1/_{10}$	1·55	0·81	NT	NT
$^1/_{30}$	1·15	0·84	2·7	1·37
$^1/_{100}$	1·0	0·95	1·95	1·20

a) PS - Positive serum ie. anti-CBA fibrosarcoma serum raised in BALB/c mice by an injection of 1×10^7 cells s.c. followed by a similar injection i.p. 7 days later.

NMS - Normal CBA mouse serum

TMS - Serum from CBA mice bearing a syngeneic fibrosarcoma

b) NT - Not tested

Note

1) Method B was more sensitive than A

2) High concentrations of mouse serum gave the highest AR

3) Target cells were CBA fibrosarcoma

OPTIMUM CONDITIONS FOR ANTIGLOBULIN ASSAY (METHOD B)

TABLE 13 VARIATION OF MOUSE SERUM CONCENTRATION

MOUSE SERUM DILUTION	PES[a] / NMS	PS[a] / NMS
1/3	1·42	1·67
1/11	1·08	1·54
1/33	1·00	1·12
1/66	0·80	1·00

TABLE 14 VARIATION OF RABBIT ANTI-MOUSE IgG CONCENTRATION

RAM IgG[a] DILUTION	PES / NMS	PS / NMS
1/30	1·47	2·30
1/90	1·50	2·16
1/150	2·04	1·76
1/220	1·16	1·29

TABLE 15 VARIATION OF ^{125}I-GLOBULIN FRACTION OF GOAT ANTI-RABBIT IgG CONCENTRATION

GAR IgG[a] CONC. (µg/WELL)	PES / NMS	PS / NMS
5	4·0	2·35
10	4·0	2·14
20	3·6	2·34
30	3·0	1·82

a) PES - Post excision serum (serum from CBA mice 14 days after amputation of palpable syngeneic tumour)
NMS - Normal CBA serum
PS - BALB/c anti-CBA fibrosarcoma serum
RAM - Rabbit anti-mouse IgG serum
GAR - Antiglobulin reagent (^{125}I-labelled globulin fraction of goat anti-rabbit IgG)

Note

Target cells were CBA fibrosarcoma.

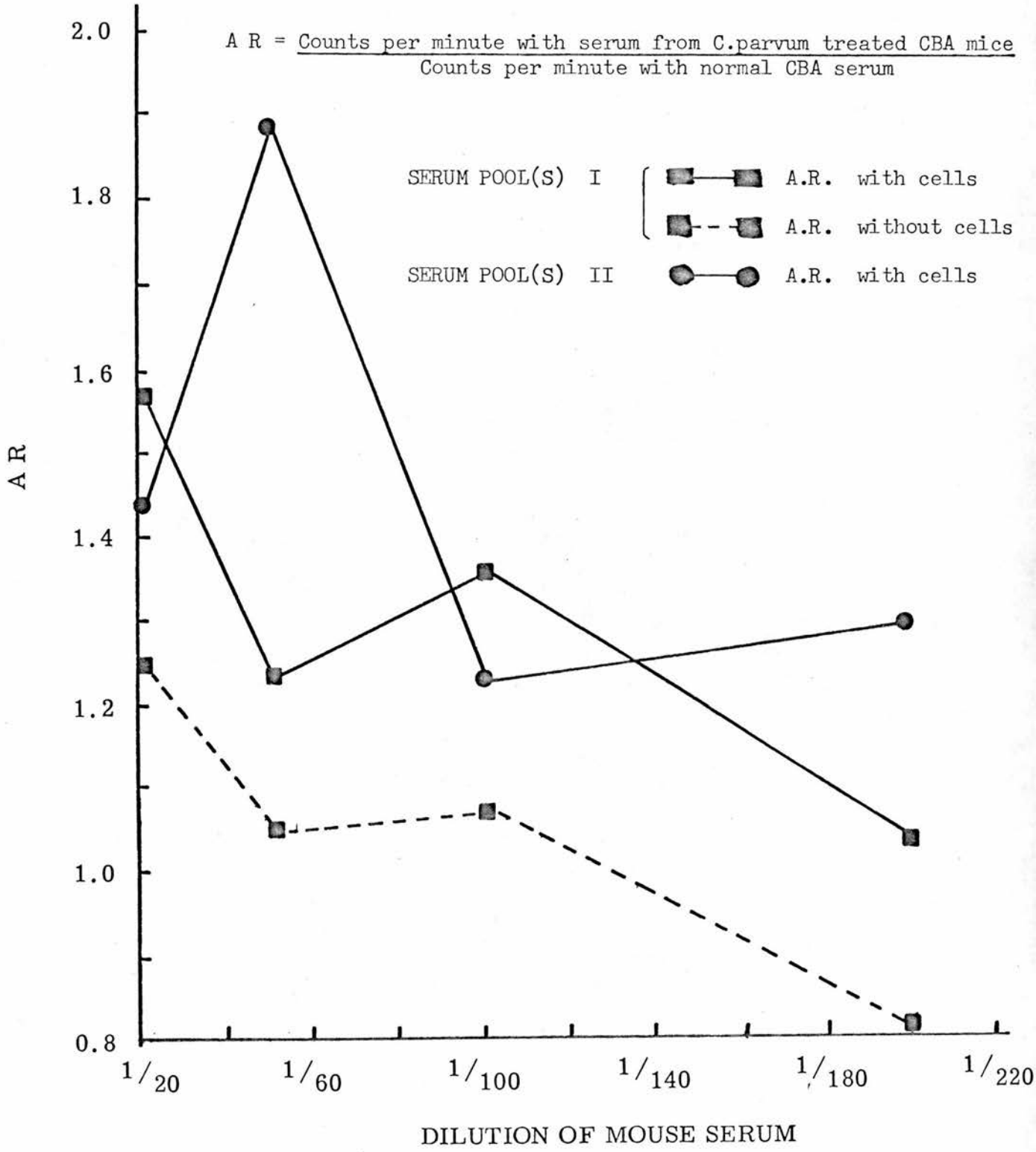
TABLE 16 EXAMINATION OF CELL LOSS DURING THE ANTIGLOBULIN ASSAY (METHOD B)

MOUSE SERUM USED IN ASSAY	% OF CELLS REMAINING AFTER ASSAY (ie. % OF ^{51}Cr CONTROL	^{125}I COUNTS (ie. MOUSE IMMUNOGLOBULIN BINDING TO CBA FIBROSARCOMA TARGET CELLS)
Normal CBA mouse serum	100	11,273
Normal CBA mouse serum (inactivated)	80	13,982
Serum from CBA mice bearing a syngeneic fibrosarcoma	87	11,767
Serum from CBA mice bearing a syngeneic fibrosarcoma (inactivated)	92	12,776

Note

Target cells subjected to the multiple washings and centrifugations (16 in all) of the assay never lost more than 20% of their original number.

Fig. 15 UPTAKE OF IMMUNOGLOBULIN BY a) CBA TUMOUR CELLS PLUS PLASTIC REACTION WELLS AND b) PLASTIC REACTION WELLS ONLY



Note (Fig. 15)

1) C.parvum (1·4 mg) injected i.p. Day 0; mice bled Day 21. Normal serum from age matched controls was used to compute the Absorption Ratio (A R).

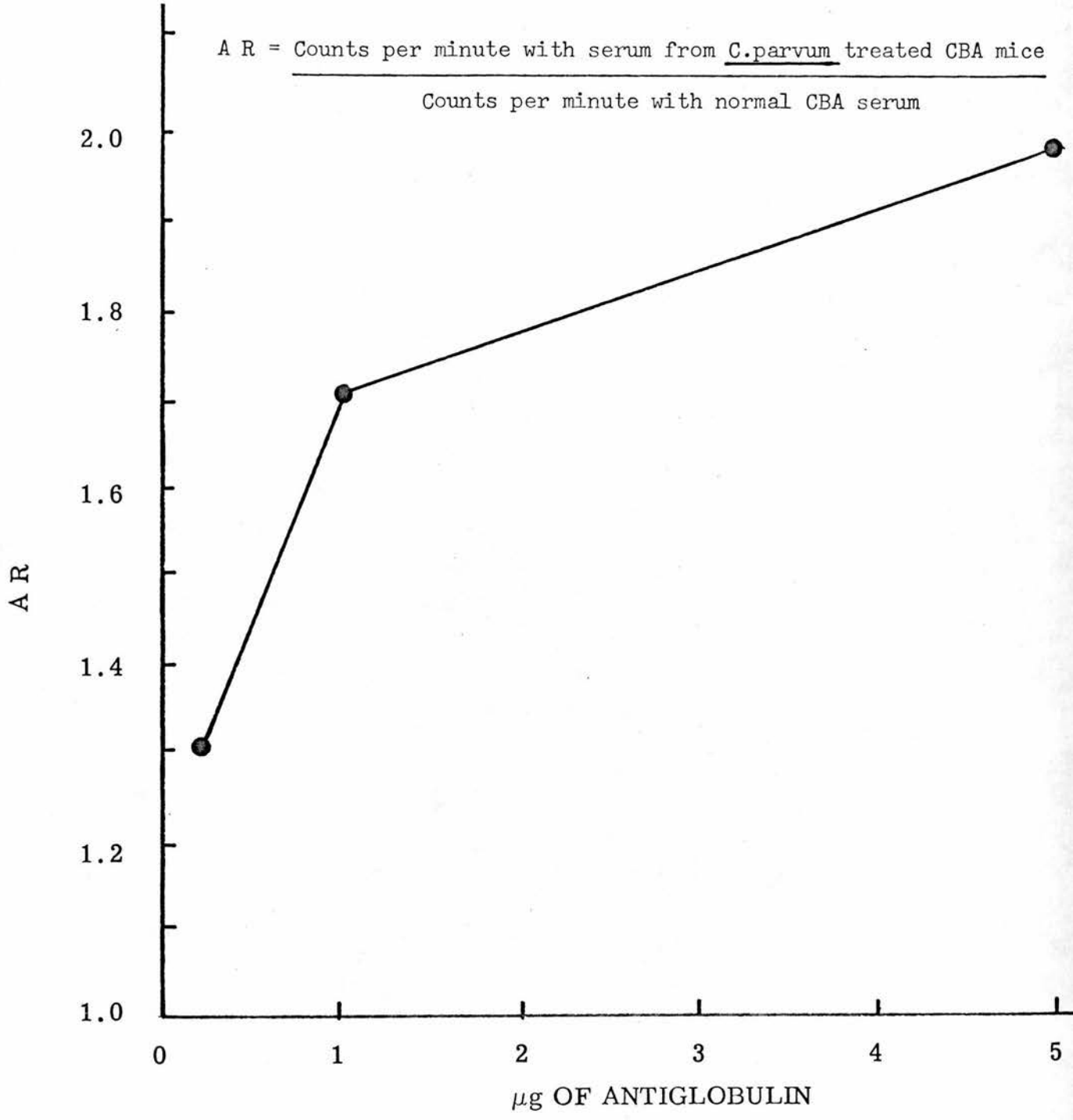
2) Two pools of serum (I and II) from C.parvum-treated mice were examined.

3) At the dilutions tested the A R with cells was always greater than without cells. Although some uptake onto plastic was observed this could be avoided by using Antiglobulin Methods C or D.

4) Antiglobulin Method B used.

Fig. 16 UPTAKE OF IMMUNOGLOBULIN BY CBA TUMOUR CELLS

(ie. CELLS REMOVED FROM THE REACTION VESSEL FOR γ-COUNTING)



Note

1) CBA mice injected with C.parvum (1·4 mg) i.p. Day 0; mice bled Day 21.

2) Antiglobulin Method C used.

3) This method was quite sensitive although there were severe limitations on the number of samples that could be processed.

TABLE 17 OPTIMUM CONDITIONS FOR ANTIGLOBULIN METHOD D : VARIATION OF CONCENTRATION OF ANTIGLOBULIN REAGENT (^{125}I-LABELLED GLOBULIN FRACTION OF GOAT ANTI-RABBIT IgG)

ANTIGLOBULIN CONC.	0·5 µg		2·5 µg		7·5 µg	
MOUSE SERUM CONC.	1/5	1/17	1/5	1/17	1/5	1/17
CBA mouse serum[a] from Group A	606[b]	424	2,688	1,607	5,203	2,699
CBA mouse serum from Group B	640 NS	507 NS	2,756 NS	2,290 P 0·01	6,372 NS	3,962 P< 0·02
CBA mouse serum from Group C	479 NS	427 NS	2,444 NS	1,685 NS	4,722 NS	2,854 NS
CBA mouse serum from Group D	699 NS	419 NS	2,624 NS	2,679 P<0·05	6,911 P<0·01	5,207 P<0·05

a) Group A = normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24. Target cells were CBA fibrosarcoma.

b) Each result is expressed as counts per minute and is the mean of triplicates. P >0·05 not significant (NS) when compared to normal serum control.

Note

1) Only high concentrations of antiglobulin reagent revealed differences in uptake of immunoglobulin from the different sera.

2) Rabbit anti-mouse IgG serum (see Materials and Methods 4a) used at 1/50.

TABLE 18 OPTIMUM CONDITIONS FOR ANTIGLOBULIN METHOD D : VARIATION OF CONCENTRATION OF ANTIGLOBULIN REAGENT (^{125}I-LABELLED GLOBULIN FRACTION OF RABBIT ANTI-GOAT IgG)

ANTIGLOBULIN CONC.	1 µg		4 µg		10 µg	
MOUSE SERUM DIL.	1/7	1/25	1/7	1/25	1/7	1/25
CBA mouse serum[a] from Group A	806[b]	633	6,261	5,325	33,648	23,802
CBA mouse serum from Group B	1,313	969	8,749	7,823	36,467	30,318
CBA mouse serum from Group C	1,186	780	8,005	6,869	38,882	37,323
CBA mouse serum from Group D	1,691	1,137	9,703	7,535	55,267	34,951

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24. Target cells were CBA fibrosarcoma.

b) Each result is expressed as counts per minute and is the mean of triplicates.

Note

1) Only low concentrations of antiglobulin reagent revealed differences in uptake of immunoglobulin from the different sera.

2) Goat anti-mouse IgG serum (Hyland Laboratories, Los Angeles, California) used at 1/50.

2. USE OF ANTIGLOBULIN ASSAY TO DETECT IMMUNOGLOBULIN REACTING IN VITRO WITH SYNGENEIC TUMOUR CELLS AFTER C.PARVUM ADMINISTRATION

a) Effect of C.parvum treatment schedule on immunoglobulin binding to tumour cells in vitro

i) Different routes of C.parvum administration in normal and tumour bearing mice

Previous studies performed in our department and elsewhere have shown that i.p. administration of C.parvum markedly inhibits the growth of a s.c. inoculum of tumour cells given three days earlier. In contrast the s.c. injection of the same dose of C.parvum at a site distant from tumour has no effect on tumour growth (3). In view of these observations we decided to compare the effect of s.c. and i.p. injection of C.parvum on the development of serum immunoglobulin capable of binding to syngeneic tumour cells in vitro in normal and tumour bearing mice. To do this the following groups of mice were set up: A - untreated control mice, B - normal mice injected s.c. (left thigh) with 1·4 mg of C.parvum on Day 3, C - normal mice injected i.p. with 1·4 mg of C.parvum on Day 3, D - mice inoculated s.c. in right thigh with 1×10^5 syngeneic fibrosarcoma cells on Day 0, E - mice as in D but also injected s.c. (left thigh) with 1·4 mg of C.parvum on Day 3, F - mice as in D but also injected i.p. with 1·4 mg of C.parvum on Day 3.

Each group was bled out on Day 24 (ie. 24 days after inoculation of tumour). The results of this experiment are summarised in Fig. 17 and Table 19.

It will be observed that in addition to inhibiting tumour growth the i.p. administration of C.parvum (Group F) resulted in the development of immunoglobulin reacting with syngeneic tumour cells in vitro. In contrast the s.c. injection of C.parvum (Group E) failed to influence tumour growth (Table 19) and had little if any effect on immunoglobulin binding to tumour cells in vitro. The i.p. administration of C.parvum to normal mice (Group C) resulted in the production of immunoglobulin binding to tumour cells in vitro whereas s.c. injection (Group B) was again without effect.

I.p. injection of C.parvum into normal and tumour bearing mice gave higher anti-C.parvum antibody titres than s.c. injection (Table 19).

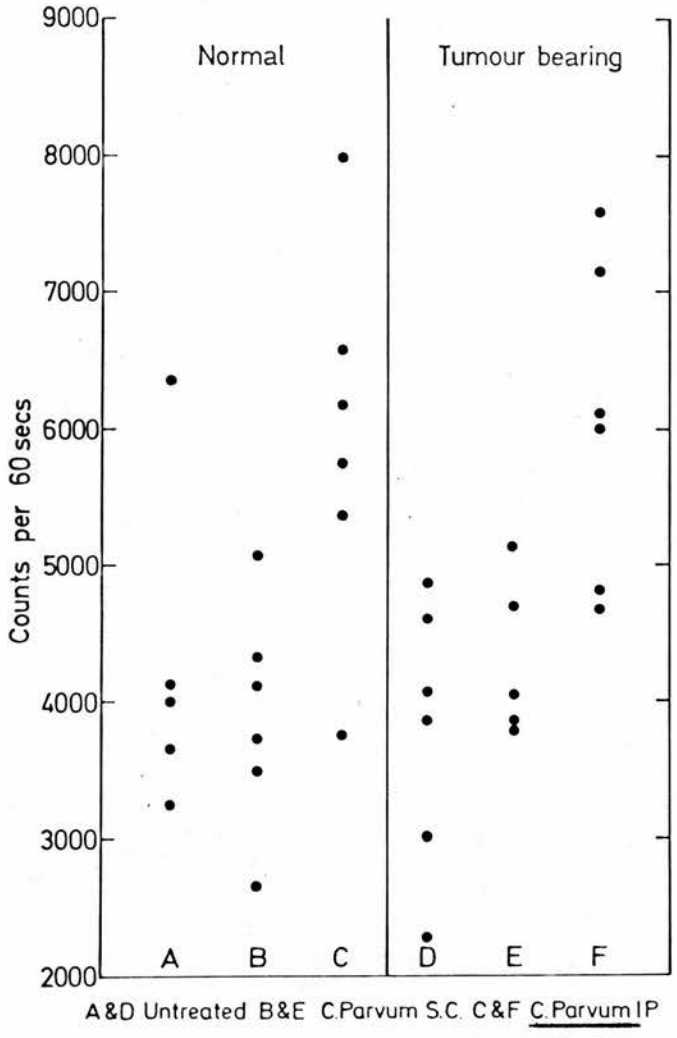
ii) Different doses of C.parvum in normal mice

In this experiment the serological changes occurring in normal mice following the administration of a range of doses of C.parvum were investigated. Mice were either untreated (Group A) or injected i.p. with 0·14 mg (Group B), 0·7 mg (Group C) or 1·4 mg (Group D) of C.parvum, and exsanguinated 7 or 21 days later. The serological changes observed are summarised in Figs. 18 and 19 and Table 20.

Immunoglobulin binding to tumour cells in vitro and antibodies to C.parvum were detectable 7 days after challenge and in general had increased substantially by Day 21 (Fig. 18 and Table 20). The binding to syngeneic tumour cells exhibited a dose dependent effect whereas the development of antibody to C.parvum

Fig. 17

EFFECT OF ROUTE OF C.PARVUM INJECTION ON UPTAKE OF IMMUNOGLOBULIN BY CBA FIBROSARCOMA CELLS IN NORMAL AND TUMOUR BEARING CBA MICE



Note

1) Tumour (1×10^5 cells) s.c. Day 0; C.parvum (1·4 mg) i.p. or s.c. on leg opposite to tumour, Day 3; mice bled Day 24.

2) In normal and tumour bearing mice i.p. injection of C.parvum gave significantly higher uptake of serum immunoglobulin than s.c. injection ($P < 0.02$).

3) In normal and tumour bearing mice s.c. injection of C.parvum did not give significantly higher uptake of immunoglobulin than untreated mice.

4) Only i.p. injection of C.parvum inhibited tumour growth ($P < 0.05$).

5) Antiglobulin Method B used.

TABLE 19

THE EFFECT OF THE ROUTE OF ADMINISTRATION ON THE RESPONSE TO AND ANTI-TUMOUR PROPERTIES OF C.PARVUM IN CBA MICE

GROUP	TREATMENT	C.PARVUM ANTIBODIES[a] (Log_2 ± S.E.)	TUMOUR DIAM. IN mm[a] (Geom. Mean ± S.E.)
A	Normal controls	1·15 ± 0·11	
B	1·4 mg C.parvum s.c. Day 3	4·35 ± 0·20	
C	1·4 mg C.parvum i.p. Day 3	8·13 ± 0·18	
D	1 x 10^5 tumour cells Day 0	1·40 ± 0·15	11·2 (9·8 - 12·8)[b]
E	1 x 10^5 tumour cells Day 0 and 1·4 mg C.parvum s.c. Day 3	3·70 ± 0·19	10·6 (9·8 - 11·4)
F	1 x 10^5 tumour cells Day 0 and 1·4 mg C.parvum i.p. Day 3	7·85 ± 0·08	8·0 (7·2 - 8·9)

a) Anti-C.parvum antibodies and tumour diameters were measured on Day 24. ie. 24 days after tumour inoculation and 21 days after C.parvum injection.

b) Values in brackets are the limits of one standard error from the geometric mean.

Note

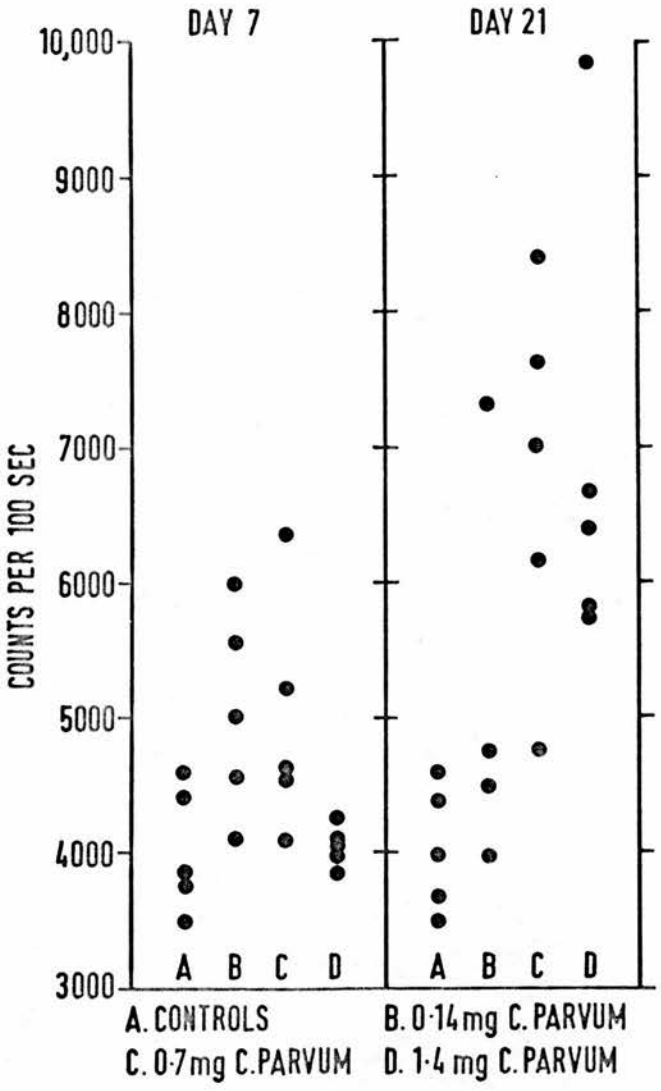
1) The antibody response to C.parvum was significantly greater following i.p. injection ($P < 0.001$) than in control mice.

2) The antibody response to C.parvum was significantly greater following i.p. injection ($P < 0.01$) than in mice treated with C.parvum s.c.

3) C.parvum administration i.p. significantly inhibits tumour growth ($P < 0.05$) while C.parvum administered s.c. fails to exert a significant effect.

Fig. 18

EFFECT OF DIFFERENT DOSES OF C.PARVUM IN CBA MICE ON THE UPTAKE OF IMMUNOGLOBULIN ONTO CBA FIBROSARCOMA CELLS



Note

1) C.parvum was injected i.p. Day 0; mice bled Day 21.

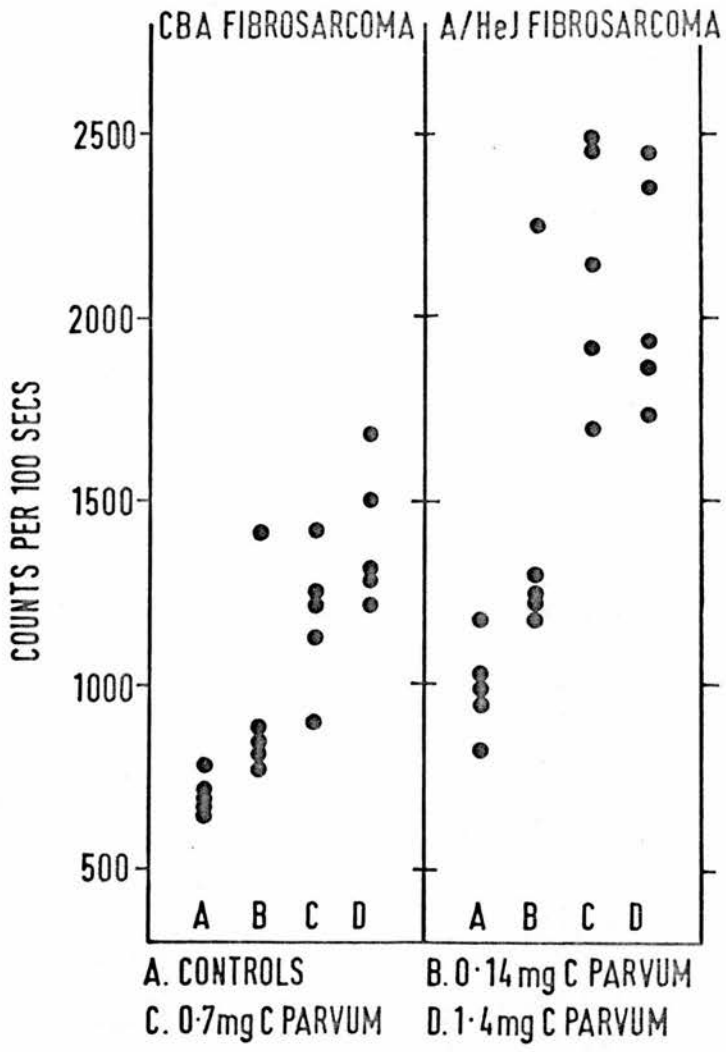
2) On Day 7 only mice treated with 0·14 mg of C.parvum gave a significant uptake of immunoglobulin ($P < 0·05$).

3) On Day 21 mice treated with 0·7 mg and 1·4 mg gave significant uptake of serum immunoglobulin ($P < 0·005$).

4) Antiglobulin Method B used.

Fig. 19

IMMUNOGLOBULIN UPTAKE FROM THE SERUM OF C.PARVUM TREATED CBA MICE ONTO CBA AND A/HeJ FIBROSARCOMA CELLS



Note

1) C.parvum was injected i.p. Day 0; mice bled Day 21.

2) The uptake of serum immunoglobulin onto both target cells was increased after administration of 0·7 and 1·4 mg of C.parvum ($P < 0·001$).

3) Antiglobulin Method B used.

TABLE 20

THE RESPONSE OF CBA MICE TO VARIOUS DOSES OF C.PARVUM INJECTED i.p.

GROUP	DOSE OF C.PARVUM (mg)	C.PARVUM ANTIBODIES (Log_2 ± S.E.)	
		Day 7	Day 21
A	None	2·07 ± 0·07	2·07 ± 0·07
B	0·1h mg	5·50 ± 0·27	8·50 ± 0·15
C	0·7 mg	5·hh ± 0·0	9·60 ± 0·28
D	1·h mg	6·15 ± 0·2h	9·28 ± 0·18

Note

The response to C.parvum was not affected by the dose administered.

did not. Further analyses were undertaken to determine the specificity of the immunoglobulin binding to CBA tumour cells in vitro. These showed that C.parvum administration resulted in the production of immunoglobulin capable of binding to both CBA and A/HeJ fibrosarcoma cells in vitro (Fig. 19).

b) Cellular basis for the production of immunoglobulin binding to syngeneic tumour cells in vitro after C.parvum administration.

i) The effect of gold salts on C.parvum activity in tumour bearing mice

A number of investigators have shown that the administration of C.parvum results in an increase in the number and activity of mononuclear phagocytes (180) and there is accumulating evidence that such cells may play an important role in tumour surveillance (133). In view of these facts we undertook studies to determine if the serological changes elicited by C.parvum could be influenced by the administration of sodium aurothiomalate, which is known to accumulate in phagocytic cells and inhibit their lysosomal and phagocytic activity (181).

In order to do this the following groups of CBA mice were set up: A - mice injected s.c. with 10^6 syngeneic fibrosarcoma cells on Day 0. B - mice injected with tumour cells as above and 1·4 mg C.parvum (i.p.) on Day 3. C - mice injected with tumour cells as above but receiving a course of injections of sodium aurothiomalate (see Fig. 20). D - mice injected with tumour cells as above on Day 0, 1·4 mg of C.parvum (i.p.) Day 3, and the standard sodium aurothiomalate treatment, The

animals were exsanguinated 21 days after C.parvum administration (Day 24) and the results of the experiment are recorded in Fig. 20 and Table 21.

It will be observed from Table 21 that the anti-tumour effect of C.parvum was completely abrogated by treatment with gold salts. Furthermore, this treatment modified the serological changes observed following C.parvum administration. For example, the development of immunoglobulin binding to syngeneic fibrosarcoma cells in vitro was completely inhibited (Fig. 20) and anti-C.parvum antibody was partially inhibited (not significantly).

ii) The effect of C.parvum in T-cell-deprived mice

A considerable amount of attention has been paid to elucidating the mechanisms whereby adjuvants exert their anti-tumour effects. For example, studies with C.parvum have shown that it exerts an anti-tumour effect against a s.c. tumour implant in B-mice, if injected i.p., but is relatively ineffective if injected directly into the same site as tumour cells (3). In the light of these observations we have undertaken studies on the serological effects of C.parvum in tumour bearing B-mice (ie. mice produced by thymectomy, X-irradiation and bone marrow reconstitution) and in congenitally athymic mice.

Experiments with tumour bearing B-mice involved the following groups:-

A. Thymectomised, irradiated, bone marrow reconstituted CBA mice (ie. B-mice) injected s.c. with 10^4 syngeneic

tumour cells on Day 0 (that is 35 days after reconstitution).

B. Mice as in group A but also injected i.p. with 0·7 mg of C.parvum on Day 3.

Further details of the thymectomies, irradiation and reconstitution procedures are recorded in Materials and Methods 1. The mice were exsanguinated on Day 25 (ie. 22 days after C.parvum administration) and the results are recorded in Fig. 21 and Table 22.

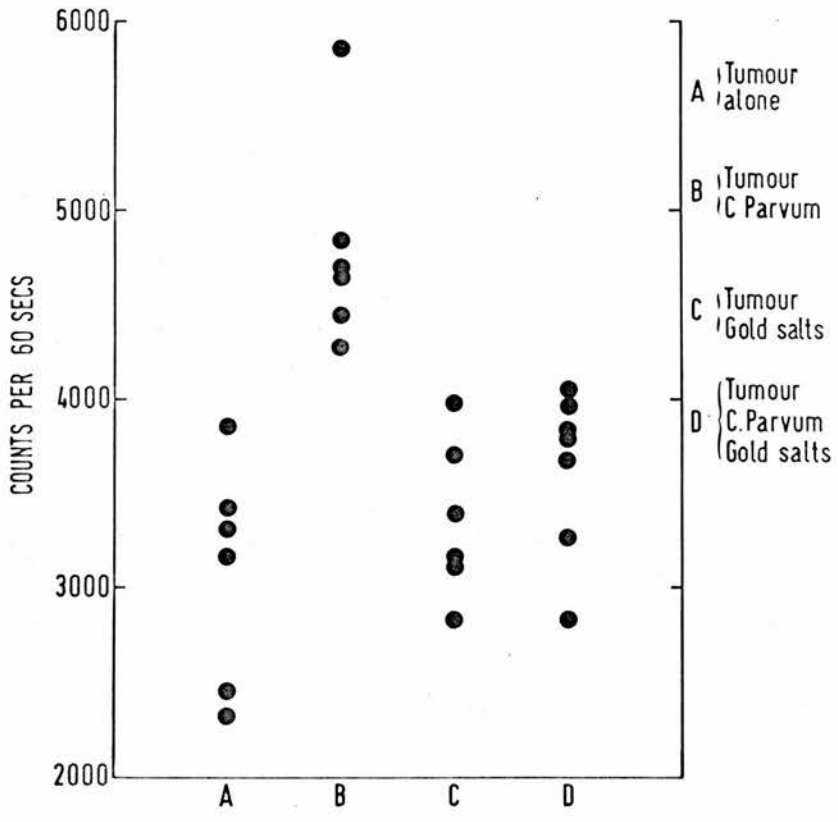
It will be observed from Table 22 that the anti-tumour effect of C.parvum was apparent in B-mice. The serological changes in this experiment were more variable than in previous experiments with intact mice, in some cases possibly due to the lack of suppressor T-cell activity. The variation was particularly apparent in Group A. Nevertheless, it should be noted that with two exceptions in Group A, the administration of C.parvum to B-mice resulted in an increase in the levels of immunoglobulin binding to tumour cells in vitro and anti-C.parvum antibody. However, in general the increases were less than in intact mice. This is illustrated in Fig. 21 which makes explicit what has hitherto been implicit, namely that there may be a correlation between immunoglobulin binding to tumour cells in vitro and anti-C.parvum antibody.

Experiments with "nude" mice involved the following groups, depending on the experiment:-

Control group: Normal BALB/c mice or heterozygous "nude" mice on

Fig. 20

EFFECT OF GOLD SALTS ON THE UPTAKE OF IMMUNOGLOBULIN BY SYNGENEIC TUMOUR CELLS IN C.PARVUM-TREATED TUMOUR BEARING CBA MICE



Note

1) Tumour (1×10^4 cells) injected s.c. Day 0; C.parvum (1·4 mg) injected i.p. Day 3; sodium aurothiomalate was administered by eight i.p. injections on alternate days starting Day 2; mice were bled on Day 24.

2) The uptake onto syngeneic tumour cells of immunoglobulin from the serum of mice treated with C.parvum-gold salts was significantly lower than mice treated with C.parvum only and was not significantly different from control mice.

3) The administration of C.parvum (Group B) significantly inhibited tumour growth ($P < 0.001$) but this effect was overcome by the administration of gold salts (Group D).

4) Antiglobulin Method B used.

TABLE 21

THE EFFECT OF GOLD SALTS ON THE RESPONSE OF CBA MICE TO C.PARVUM AND ITS ANTI-TUMOUR ACTIVITY

GROUP	TREATMENT	C.PARVUM ANTIBODIES[a] (Log_2 ± S.E.)	TUMOUR DIAM. IN MM[a] (Geom. Mean ± S.E.)
A	1×10^4 tumour cells (s.c.) on Day 0	3·73 ± 0·33	14·1 (13·6 - 14·6)
B	1×10^4 tumour cells (s.c.) on Day 0 and 1·4 mg C.parvum (i.p.) on Day 3	8·95 ± 0·17	10·7 (10·5 - 11·0)[b]
C	1×10^4 tumour cells (s.c. on Day 0 and repeated (i.p.) injection of 1 mg Sodium Aurothiomalate commencing on Day 2	3·31 ± 0·16	13·8 (13·2 - 14·5)
D	1×10^4 tumour cells (s.c.) on Day 0, 1·4 mg C.parvum (i.p.) on Day 3 and repeated (i.p.) injection of Sodium Aurothiomalate commencing Day 2	6·78 ± 0·34	12·8 (12·3 - 13·9)

a) Anti-C.parvum antibodies and tumour diameters were measured on Day 24, ie. 24 Days after tumour inoculation and 21 days after C.parvum injection.

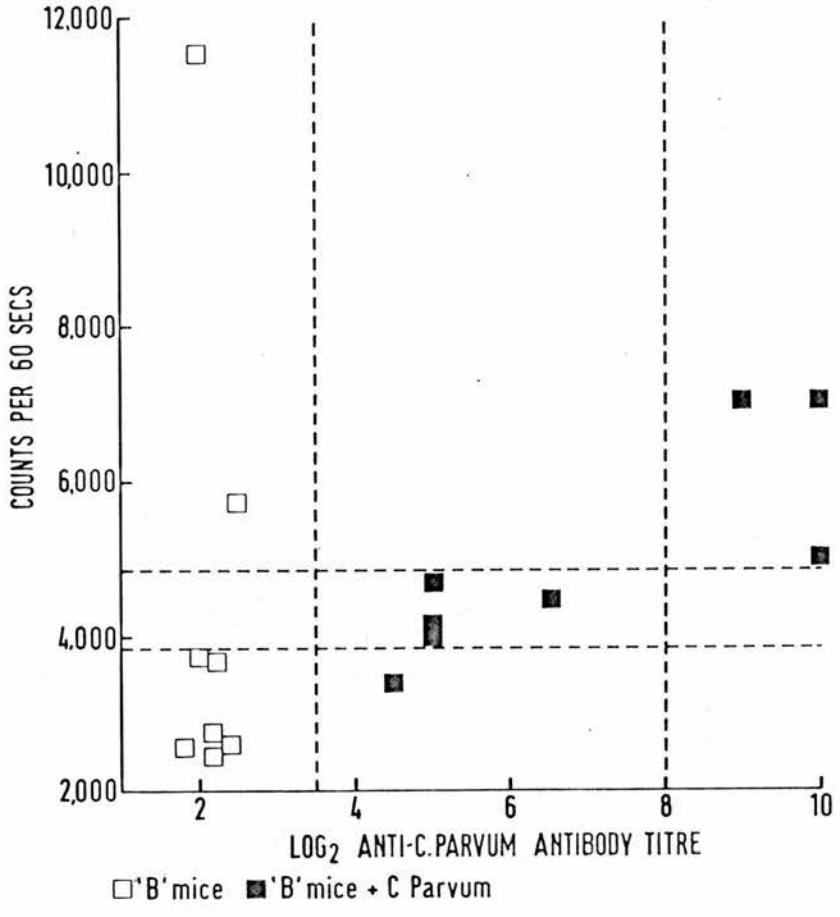
b) Values in brackets are the limits of one standard error from the geometric mean.

Note

The administration of C.parvum (Group B) significantly inhibits tumour growth ($P < 0·001$) but this effect is overcome by the administration of gold salts (Group D). This treatment however does not significantly inhibit the formation of antibodies to C.parvum.

Fig. 21

CORRELATION BETWEEN IMMUNOGLOBULIN TAKEN UP BY CBA FIBROSARCOMA CELLS AND ANTI-C.PARVUM ANTIBODY IN THYMUS-DEPRIVED CBA MICE



Note

1) Tumour (1×10^4 cells) injected s.c. Day 0; C.parvum (0·7 mg) injected i.p. Day 3; mice bled Day 25.

2) In general the administration of C.parvum to 'B'-mice resulted in serological changes of lesser magnitude than in previous studies with intact mice. Only three mice gave responses comparable to previous experiments.

3) C.parvum inhibited tumour growth in 'B'-mice ($P < 0.005$). Again the effect seemed less than in intact mice.

4) Antiglobulin Method B used.

TABLE 22 THE EFFECTS OF C.PARVUM IN TUMOUR BEARING[a] T-CELL DEFICIENT ('B') MICE

ASSAY		WITHOUT C.PARVUM	WITH C.PARVUM
Tumour diameter (mm)[b] (Geom. mean ± S.E.)		14·4 (13·7 - 15·2)	11·2 (10·7 - 11·8)
Anti-tumour antibody[c]		See Fig. 21	
C.parvum antibodies[c] (Log_2 ± S.E.)		2·1 ± 0·1	7·1 ± 0·9
IgM	per cent standard serum	55·4 ± 7·0	106·9 ± 11·7
IgA	per cent standard serum	501·8 ± 142·2[d]	1183·3 ± 109·1
IgG_1	per cent standard serum	162·6 ± 21·3	189·8 ± 26·7
IgG_{2a}	per cent standard serum	15·2 ± 2·1	30·8 ± 3·0
IgG_{2b}	per cent standard serum	58·2 ± 2·2	106·8 ± 11·4

a) Mice challenged on Day 0 with 1 x 10^4 tumour cells s.c., 35 Days after reconstitution with bone marrow cells. C.parvum (0·7 mg) injected i.p. Day 3.

b) Tumour measured 23 days after tumour transplantation.

c) All serological assays performed on serum taken 25 days after tumour transplantation.

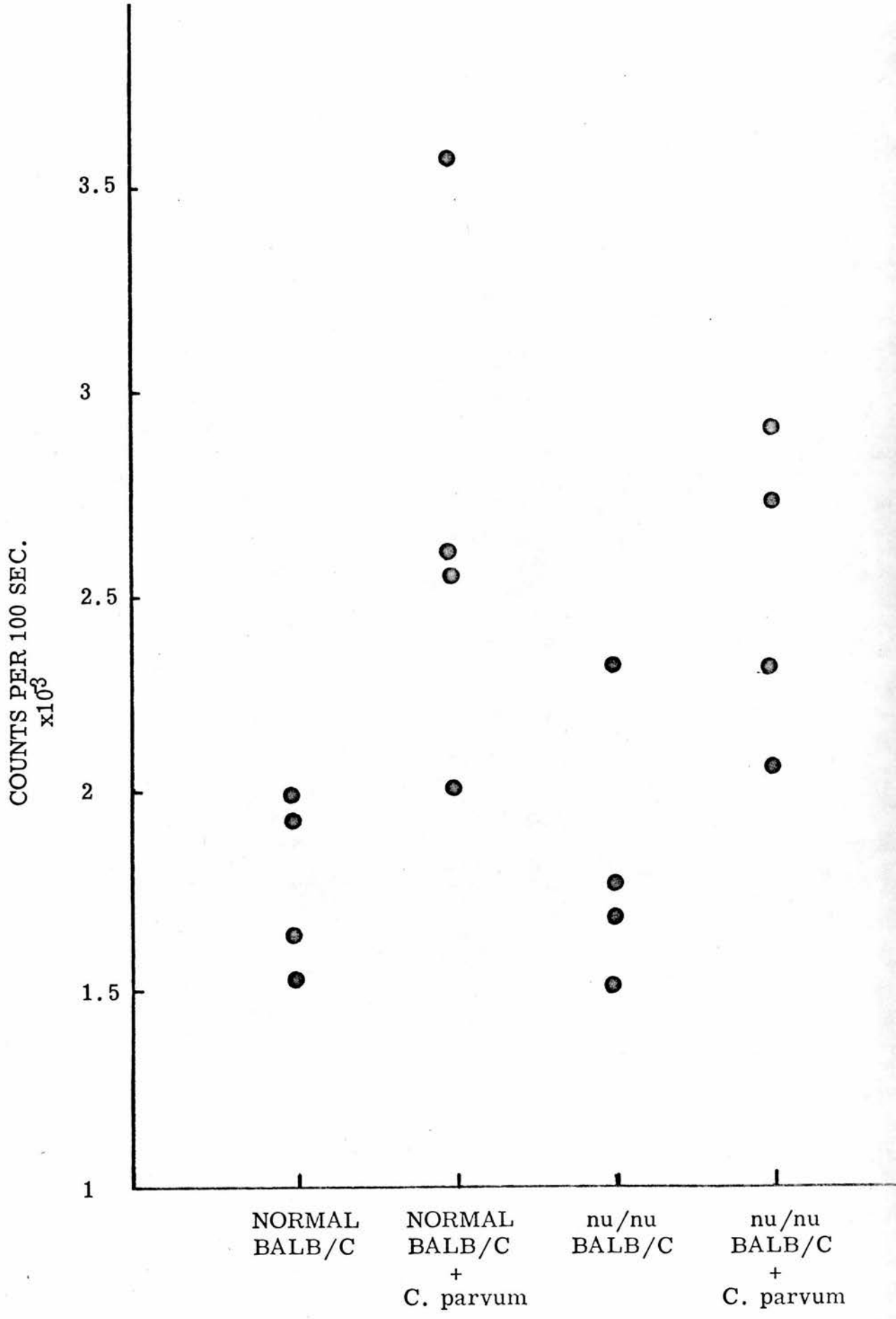
d) A new normal serum standard was used in these assays. This appears to be deficient in IgA.

Note

1) Tumour inhibition after C.parvum treatment significant at $P < 0·005$

2) With the exception of the IgG_1 values all other measurements of immunoglobulin levels in C.parvum-treated animals are significantly different than in the untreated group.

Fig. 22 EFFECT OF C.PARVUM IN CONGENITALLY ATHYMIC MICE ON SERUM IMMUNOGLOBULIN BINDING TO CBA TUMOUR CELLS



Note (Fig. 22)

1) C.parvum (0·7 mg) injected i.p. Day 0; mice bled out on Day 18.

2) The athymic trait was inserted into the Balb/c background.

3) After C.parvum injection normal ($P < 0·025$) and homozygous ($P < 0·05$) "nude" mice had higher levels of immunoglobulin binding to CBA fibrosarcoma cells than untreated controls.

4) Antiglobulin Method B used.

TABLE 23 SEROLOGICAL CHANGES IN "NUDE" MICE AFTER ADMINISTRATION OF C.PARVUM

Serological parameter[a] measured (Mean ± 1 S.E.)	Heterozygous "nude" mice on a BALB/c background			Homozygous "nude" mice on a BALB/c background		
	untreated	C.parvum treated[b]		untreated	C.parvum treated[b]	
		i.v.	i.p.		i.v.	i.p.
Immunoglobulin binding to CBA fibrosarcoma cells in vitro (counts per min.)	2051 (1823-2307)	2509 (2237-2813)	3491** (3280-3715)	2226 (1834-2696)	3482* (3274-3703)	2576 (2084-3185)
Serum immunoglobulin class and sub-class levels (mg/dl)						
IgM	38·4 (35·6-41·4)	70·1** (65·0-75·7)	84·8*** (78·6-91·5)	59·1 (56·3-62·0)	75·6** (72·0-79·4)	57·9 (53·2-63·0)
IgA	8·8 (8·4-9·1)	6·8* (6·3-7·4)	8·2 (7·6-8·7)	4·4 (3·9-5·0)	6·1 (5·5-6·0)	5·6 (5·1-6·2)
IgG_1[c]	30·6 (26·3-35·7)	74·6 (63·7-87·5)	97·8*** (88·0-108·7)	10·8 (4·5-26·4)	24·8 (10·2-60·3)	29·2 (10·0-85·2)
IgG_{2a}[c]	61·9 (56·2-69·3)	174·9** (141·4-216·2)	272·5*** (236·8-313·7)	6·8 (3·0-15·5)	54·0* (28·7-101·4)	39·0 (16·0-95·0)
IgG_{2b}	11·1 (9·8-12·6)	22·0** (18·8-25·7)	40·0*** (33·9-47·3)	18·7 (15·5-22·6)	21·5 (17·4-26·5)	24·3 (19·2-30·9)
Anti-C.parvum antibody (log_2 reciprocal titre)	3·67 ± 0·17	10·15 ± 0·30***	10·9 ± 0·3***	3·0 ± 0·2	5·4 ± 0·3***	4·85 ± 0·13***

a) The mean value for each parameter measured was calculated from at least 5 individual mice

b) 0·7 mg C.parvum administered Day 0. Mice bled Day 21.

c) There was wide variations in the IgG_1 and IgG_{2a} levels in untreated and treated homozygous "nude" mice.

* $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$

a BALB/c background, either untreated or injected with 0·7 mg C.parvum i.p. or i.v.

Experimental group: Homozygous "nude" mice on a BALB/c background, either untreated or injected with 0·7 mg C.parvum i.p. or i.v. Mice were bled out 18 - 21 days after C.parvum injection.

It can be seen from Fig. 22 and Table 23 that administration of C.parvum to "nude" mice resulted in a significant increase in the levels of immunoglobulin binding to allogeneic (CBA) fibrosarcoma cells. In addition anti-C.parvum antibody titres were significantly elevated.

It should be noted that the highest values for the immunoglobulin binding to CBA fibrosarcoma cells in vitro after C.parvum injection were similar in homozygous and heterozygous "nude" mice. In contrast, the anti-C.parvum antibody titres in heterozygous "nude" mice were higher than in homozygous "nude" mice (Table 23).

c) Effect of C.parvum on immunoglobulin bound to tumour cells in vivo

In view of the observation by Ran et al. (182) that tumour cells may be coated in vivo with immunoglobulin, experiments were set up to determine if C.parvum therapy affected the amount of immunoglobulin bound to tumour cells in vivo in our system. To this end an indirect antiglobulin assay with rabbit anti-mouse IgG and ^{125}I-labelled globulin fraction of goat anti-rabbit IgG was used to detect immunoglobulin on freshly excised, mechanically disrupted CBA fibrosarcoma cells. Antiglobulin method B was used to detect immunoglobulin capable of binding to CBA fibrosarcoma cells in vitro in the serum of autochthonous CBA

mice (ie. those mice examined for *in vivo* bound immunoglobulin).

These studies revealed that the amount of immunoglobulin bound *in vivo* was increased after *C.parvum* administration (Fig. 23 and Table 24). Furthermore, there was a correlation between immunoglobulin bound *in vivo* and *in vitro* to CBA fibrosarcoma cells (Fig. 24). This is further illustrated by examining the ratios of *in vivo* bound to *in vitro* bound immunoglobulin. Thus, the ratios were not significantly different in the *C.parvum*-treated group or the untreated group, indicating a direct relationship between *in vivo* and *in vitro* bound immunoglobulin. It should be noted that these experiments do not reveal whether the immunoglobulin detected *in vivo* was bound via the Fab or Fc region.

d) *Effect of different strains of C.parvum, in normal and tumour bearing mice, on immunoglobulin binding to syngeneic tumour cells in vitro.*

It was of interest to see if other strains of *C.parvum* elicited the production of serum immunoglobulin capable of binding to syngeneic fibrosarcoma cells. To investigate this *C.parvum* CN 6134, *C.parvum* 10470 (*P.freudenreichii*) and *C.parvum* 10387 were injected into CBA mice and their ability to inhibit tumour growth and to elicit immunoglobulin binding to syngeneic tumour cells *in vitro* was examined.

All of the strains injected into normal mice induced small but significant increases in serum immunoglobulin capable of binding to syngeneic tumour cells *in vitro* (Fig. 25). In tumour bearing mice strain CN 6134 and 10470 again evoked small but significant increases in serum immunoglobulin capable of binding to syngeneic tumour cells

TABLE 24 IN VIVO AND IN VITRO TUMOUR BOUND IMMUNOGLOBULIN IN C.PARVUM-TREATED TUMOUR BEARING MICE[a]

PARAMETER MEASURED [b]	UNTREATED	C.PARVUM (day +3)	P- VALUE
Tumour diameter (mm)	18·5 (17·4-19·8)	13·7 (13·1-14·4)	0·005→0·01
In vivo tumour bound immunoglobulin (counts/min/5 x 10^4 cells)	17,658 (16,450-18,954)	23,096 (22,298-23,922)	<0·001
In vitro tumour bound immunoglobulin (counts/min)	5,456 (5148-5784)	8,202 (7866-8552)	≏0·005

a) 1·4 mg of C.parvum administered (i.p.) 3 days after s.c. transplantation of 1 x 10^5 viable CBA fibrosarcoma cells.

b) All parameters measured 24 days after tumour transplantation.

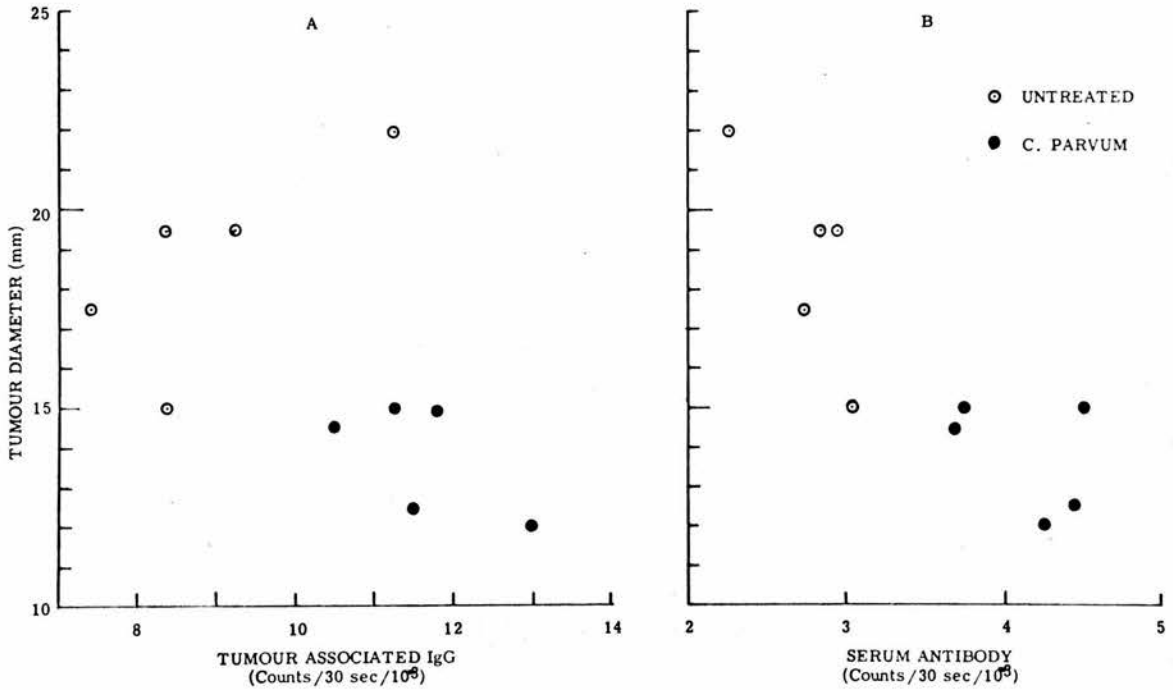
Note

1) C.parvum administration significantly inhibited tumour growth.

2) C.parvum administration resulted in significant increases in immunoglobulin bound to CBA fibrosarcoma cells, both in vivo and in vitro.

3) Antiglobulin method B used.

Fig. 23 DETECTION OF IMMUNOGLOBULIN ON THE TUMOUR CELL SURFACE AND IN AUTOCHTHONOUS SERUM



Note

1) CBA fibrosarcoma (1×10^5 cells) injected s.c. Day 0; C.parvum (1·4 mg) injected i.p. Day 3; on Day 24 mice were bled out and a cell suspension was made from each tumour, keeping the temperature at 4°C.

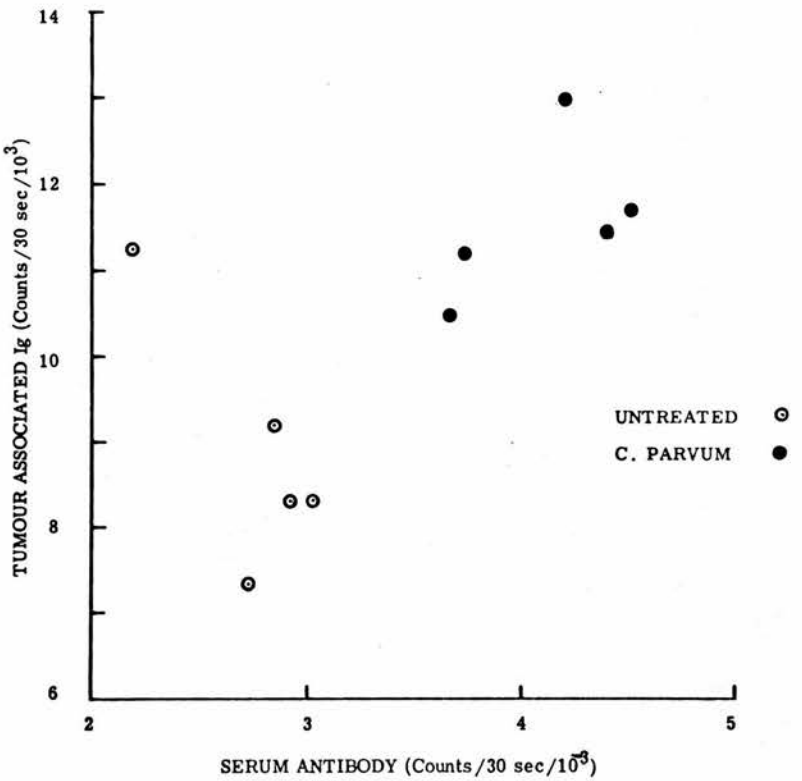
2) Greater amounts of immunoglobulin were found on the tumour cell surface in C.parvum-treated mice ($P < 0{\cdot}001$) than in untreated mice.

3) Higher levels of serum immunoglobulin binding to CBA fibrosarcoma cells were found in the sera of C.parvum-treated mice ($P = 0{\cdot}005$) than in untreated mice.

4) C.parvum inhibited tumour growth ($P < 0{\cdot}01$).

Fig. 24

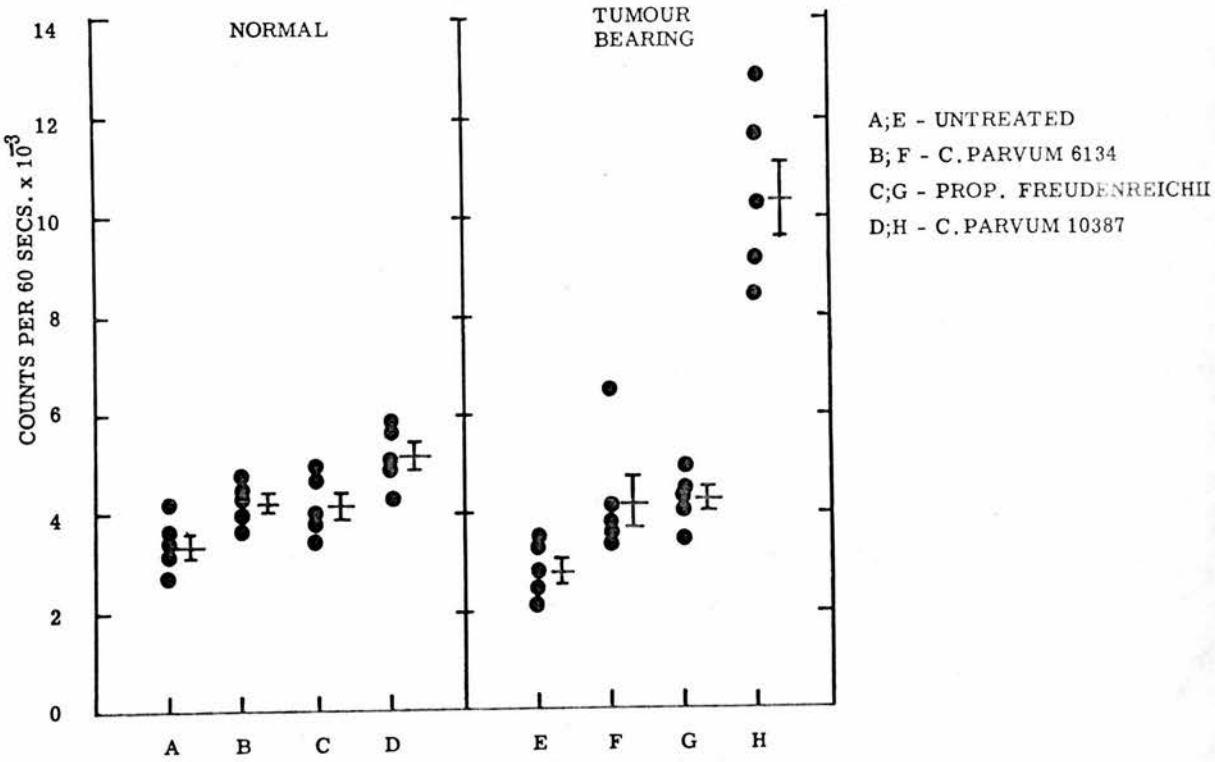
CORRELATION BETWEEN IMMUNOGLOBULIN DETECTED ON THE TUMOUR CELL SURFACE AND IN AUTOCHTHONOUS SERUM



Note

With one conspicuous exception there is a correlation between the amount of immunoglobulin in mouse serum capable of binding to CBA fibrosarcoma cells, and the amount of immunoglobulin detected directly on the tumour cell surface.

Fig. 25 EFFECT OF DIFFERENT STRAINS OF C.PARVUM IN NORMAL AND TUMOUR BEARING CBA MICE ON UPTAKE OF IMMUNOGLOBULIN ONTO SYNGENEIC FIBROSARCOMA CELLS



Note

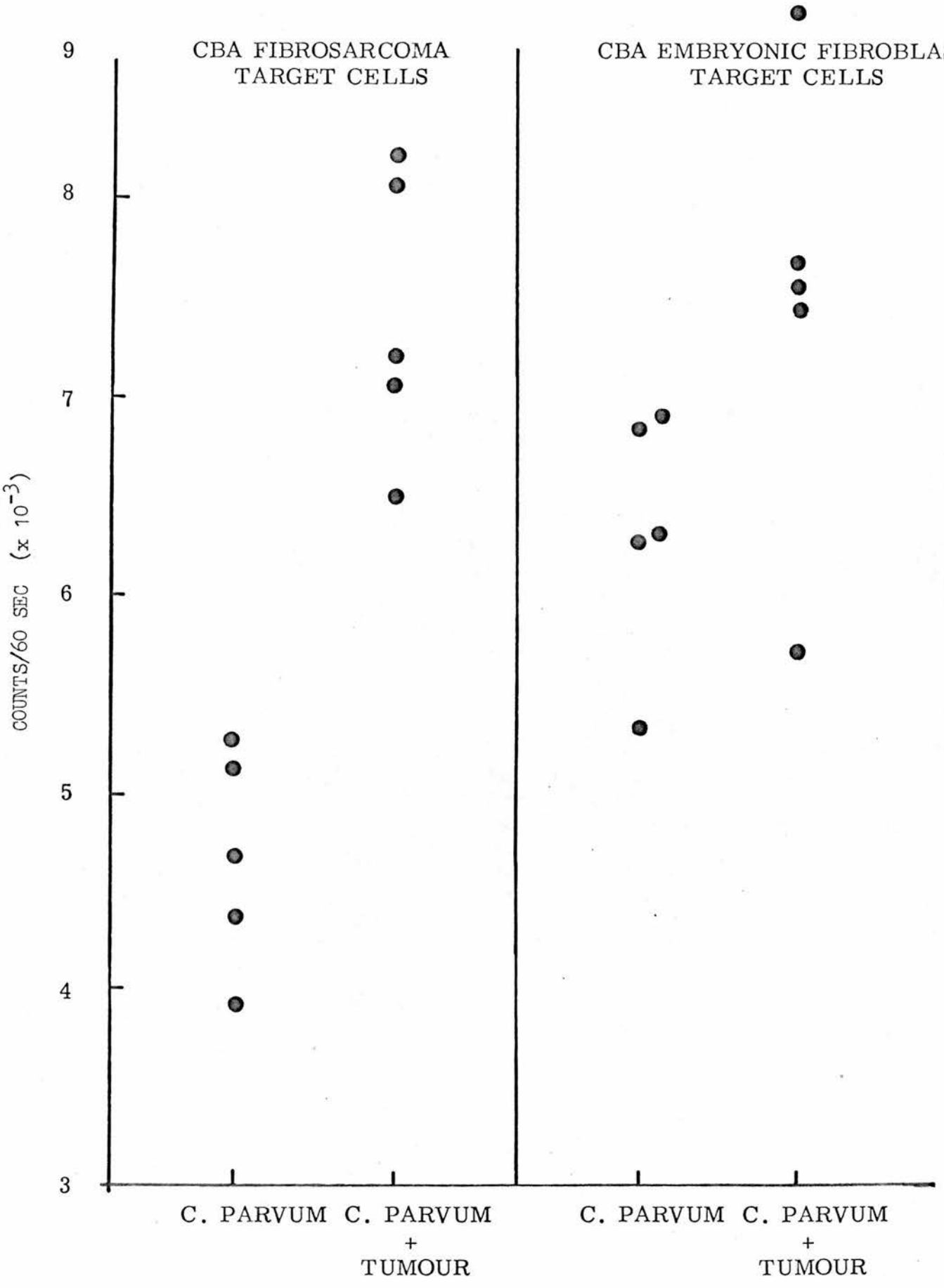
1) Tumour (1×10^5 cells) injected s.c. Day 0; C.parvum (strain 6134 and 10470 (P. freudenreichii) 1·4 mg, strain 10387 0·7 mg) injected i.p. Day 3; mice bled Day 24.

2) In normal mice all strains of C.parvum resulted in a small but significant increase ($P < 0.05$) in levels of serum immunoglobulin binding to CBA fibrosarcoma cells. In tumour bearing mice the increase was most significant for C.parvum 10387 ($P < 0.001$).

3) C.parvum strains 6134 and 10387 inhibited tumour growth ($P \leq 0.01$). Strain 10470 was ineffective.

4) Antiglobulin Method B used.

Fig. 26 SPECIFICITY OF ANTI-TUMOUR ANTIBODY PRODUCED BY C.PARVUM 10387 IN CBA MICE BEARING A SYNGENEIC FIBROSARCOMA



Note (Fig. 26)

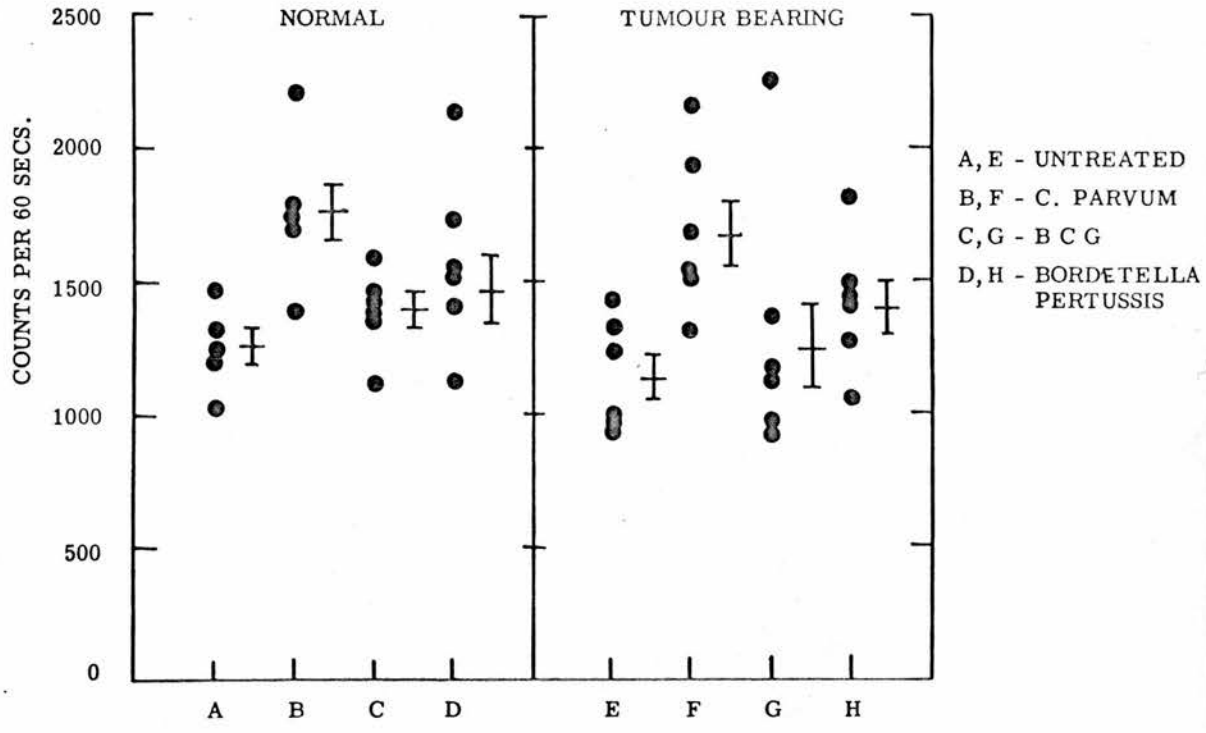
1) Tumour (1×10^5 cells) injected s.c. Day 0; C.parvum (strain 10387) 0·7 mg injected i.p. Day 3; mice bled Day 24.

2) The uptake of immunoglobulin onto CBA fibrosarcoma cells from the serum of mice treated with tumour and C.parvum 10387 was greater than for mice treated with C.parvum 10387 alone ($P < 0{\cdot}005$). The uptake onto CBA embryonic fibroblasts was not significantly different.

3) C.parvum strain 10387 inhibited tumour growth ($P < 0{\cdot}001$).

4) Antiglobulin Method B used.

Fig. 27 EFFECT OF DIFFERENT ADJUVANTS IN NORMAL AND TUMOUR BEARING CBA MICE ON UPTAKE OF IMMUNOGLOBULIN ONTO CBA FIBROSARCOMA CELLS



Note

1) Tumour (1×10^5 cells) injected s.c. Day 0; adjuvants (C.parvum 0·7 mg, B.C.G. 9×10^5 organisms, B. pertussis 4×10^9 organisms) injected i.p. Day 3; mice bled Day 24.

2) In normal and tumour bearing mice administration of C.parvum caused significant increases ($P < 0{\cdot}01$) in the levels of serum immunoglobulin binding to CBA fibrosarcoma cells, while B. pertussis caused a significant increase ($P < 0{\cdot}05$) in tumour bearing mice. B.C.G. was ineffective in normal and tumour bearing mice, as were Freund's complete and incomplete adjuvant (0·1 ml). (Not shown)

3) C.parvum ($P < 0{\cdot}001$) and B. pertussis ($P < 0{\cdot}05$) caused significant inhibition of tumour growth.

4) Antiglobulin Method B used.

in vitro, whereas strain 10387 induced a large increase. The difference between normal and CBA fibrosarcoma bearing mice treated with C.parvum 10387 suggested that part of the response might be due to unique tumour antigens. Fig. 26 indicates that this may be the case because the relative uptake onto CBA fibrosarcoma cells in vitro from the serum of mice treated with C.parvum 10387 and tumour was greater than the uptake onto CBA embryo cells. The relative uptake is defined here as the uptake compared with mice treated with C.parvum 10387 only.

e) Effect of different adjuvants, in normal and tumour bearing mice, on immunoglobulin binding to syngeneic tumour cells in vitro.

It was also of interest to see if adjuvants, ostensibly unrelated to C.parvum, could elicit immunoglobulin capable of binding to syngeneic fibrosarcoma cells. Thus, normal and CBA fibrosarcoma bearing mice were injected with C.parvum, BCG and B.pertussis, and their ability to inhibit tumour growth and to elicit immunoglobulin capable of binding to syngeneic fibrosarcoma cells in vitro was measured.

At the stated doses the C.parvum protocol produced the most dramatic inhibition of tumour growth and the highest levels of immunoglobulin capable of binding to syngeneic tumour cells (Fig. 27). While B.pertussis produced minor effects on both of these parameters, BCG was ineffective.

f) Characteristics of serum immunoglobulin binding to syngeneic fibrosarcoma cells in vitro

For these experiments pools of serum were obtained from normal CBA mice, C.parvum-treated mice, CBA mice bearing a syngeneic fibro-

sarcoma, and CBA mice bearing a syngeneic fibrosarcoma treated with C.parvum. To investigate the class of immunoglobulin binding to syngeneic fibrosarcoma cells in vitro the above serum pools were separated on G-200 sephadex and the fractions examined by antiglobulin Methods B and D. In addition the anti-C.parvum antibody titres of the fractions were measured.

It can be seen (Table 25) that antiglobulin method D was more sensitive than B as it revealed a 7S as well as a 19S response in C.parvum-treated tumour bearing mice. The 19S response correlated with high anti-C.parvum antibody titres whereas the 7S did not.

Another approach to this problem was to use antisera specific for mouse class and sub-class immunoglobulins in the antiglobulin assay. The results of such an experiment are presented in Tables 26 and 27. It can be seen that mice treated with C.parvum had increased levels of IgM and IgG_{2a} (on the borderline of significance taking a 30% increase as significant) capable of binding to syngeneic fibrosarcoma cells in vitro. Tumour bearing mice had an increase of IgG_1, and tumour bearing mice treated with C.parvum an increase of all classes and sub-classes except IgA. The results using G-200 sephadex fractions and antisera specific for mouse class and sub-class immunoglobulins were in close agreement.

g) Specificity of serum immunoglobulin binding to syngeneic fibrosarcoma cells in vitro

There are several possible explanations for the presence in serum of C.parvum-treated mice of increased levels of immunoglobulin capable of interacting with syngeneic fibrosarcoma cells. These include:

a) the production of increased levels of cytophilic IgG which binds to infiltrating host cells; b) the production of antibody against antigenic determinants shared by C.parvum and mammalian cells; c) an increased production or a de novo synthesis of autoantibody; d) an increase in the level of pre-existing anti-tumour antibody; e) the production of antibody against unique tumour antigens. Some of these mechanisms were investigated in the following experiments.

i) Blocking of immunoglobulin uptake with aggregated human IgG

It might be expected a priori that uptake onto in vitro cultured cells would not be via Fc-receptors (and therefore would not be blocked with aggregates) as the number of Fc/bearing -receptor cells in a fresh tumour cell suspension decreases rapidly in in vitro culture conditions (102). This point was confirmed by preincubating the in vitro cultured target cells with aggregated human IgG. After washing, the cells were used in the standard antiglobulin assay using either Method B (Table 28), or Method D (Tables 30, 31). It can be seen that only when using fresh spleen cells as targets did preincubation with aggregates cause inhibition of immunoglobulin uptake.

ii) Uptake of immunoglobulin from whole serum onto malignant and non-malignant cells

To obtain some insight into the antigenic determinants that the antibody detected by the antiglobulin assay was reacting against, it was necessary to use non-malignant target cells. Therefore, CBA embryonic fibroblasts (in vitro cultured) and spleen cells (freshly prepared) were used as target cells in the

antiglobulin assay, concurrently with the usual CBA fibrosarcoma cells.

It can be seen that immunoglobulin, from the unfractionated serum of mice treated with C.parvum, bound to CBA fibrosarcoma cells and CBA embryonic fibroblasts to about the same extent (Fig. 28). Furthermore, the uptake onto spleen cells was greater than onto fibrosarcoma cells. This latter phenomenon was dependent on the number of target cells (Fig. 29, expt. 1) and whether they contained glass-adherent cells (Fig. 29, expt. 2).

iii) Uptake of immunoglobulin from G-200 serum fractions onto malignant and non-malignant target cells.

In section 2f it can be seen that CBA mice, bearing a syngeneic fibrosarcoma and treated with C.parvum, are capable of a complex antibody response to CBA fibrosarcoma cells. It was therefore of interest to see if the same specificity towards target cells was exhibited by both the 19S and 7S response. To investigate this the major immunoglobulin containing G-200 fractions were tested in the antiglobulin assay (Method D) against CBA embryonic fibroblasts, CBA spleen cells, and A/HeJ tumour cells. In each experiment CBA fibrosarcoma cell targets were also tested for a direct comparison.

The results in section 2g(ii) indicated that whole serum from C.parvum-treated normal mice did not exhibit specificity for CBA fibrosarcoma cells when compared with syngeneic embryonic fibroblasts or spleen cells. Indeed, the relative uptake onto normal CBA spleen cells was greatest. This appears to be reflected in

the 19S serum fractions as this fraction of serum from C.parvum-treated mice exhibited the greatest uptake (relative to the 19S fraction of normal serum) when CBA normal kidney fibroblasts were used as target cells (Table 29).

A more interesting point is whether C.parvum promotes an in vivo response to tumour antigens, either common or unique. To examine this, serum from mice treated with C.parvum plus CBA fibrosarcoma cells was compared with serum from mice treated with C.parvum alone. Thus, it can be seen that the 7S fraction of serum from mice treated with C.parvum plus CBA fibrosarcoma cells exhibited a greater relative uptake onto CBA fibrosarcoma cells than onto any of the other cells tested (Tables 29 - 31). This data is summarised in Table 32.

iv) Effect of absorption on uptake of serum immunoglobulin onto CBA fibrosarcoma cells in vitro

Another method of assessing the specificity of an antibody reaction is absorption with agents which might be expected to possess relevant antigenic determinants. Therefore, sera from CBA fibrosarcoma bearing mice and C.parvum-treated CBA fibrosarcoma bearing mice were absorbed with either CBA fibrosarcoma cells, CBA spleen cells, or C.parvum. Using cells, 1 ml of each serum (diluted 1/10) was absorbed three times for 1 hour at 4°C with 6×10^6 methanol/acetic acid-fixed cells. Using C.parvum, 1 ml of each serum (diluted 1/10) was absorbed 3 times for 30 min. at 37°C with 1/10 packed cell volume of organisms.

These studies revealed that the serum immunoglobulin capable of

TABLE 25

UPTAKE ONTO CBA FIBROSARCOMA CELLS OF G-200 SEPHADEX FRACTIONS OF SYNGENEIC SERUM, USING ANTIGLOBULIN METHODS B AND D

METHOD B

SERUM FROM GROUP[a]	SERUM FRACTION			
	19S	10S	7S	4·5S
A	639[b]	427	403	267
B	927[c] (7·9)	321 (2·6)	485 (5·9)	226 (3·4)
C	499	349	383	265
D	1,241 (8·4)	349 (3·4)	463 (4·6)	277 (2·9)

METHOD D

SERUM FROM GROUP	SERUM FRACTION			
	19S	10S	7S	4·5S
A	13,720	11,627	13,851	9,416
B	17,037 (8·8)	12,841 (7·3)	10,178 (6·8)	8,133 (1·8)
C	9,792	12,772	13,609	10,699
D	16,910 (8·5)	17,355 (7·0)	27,043 (6·0)	10,815 (2·8)

MEDIUM ONLY CONTROL 8,581

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Results are expressed as counts per 60 sec. (Method B) and 300 sec. (Method D).

c) Anti-C.parvum antibody titres in parentheses. In mice not treated with C.parvum the titres were always < 3.

Note (Table 25)

1) It is not valid to compare results expressed as absolute counts per unit time: experimental groups must be compared with the relevant mouse serum controls.

2) Uptake of immunoglobulin was similar in both methods; however, only Method D gave an increased uptake from 7S fractions in Group D.

3) The uptake of immunoglobulin onto CBA fibrosarcoma cells corresponded to the anti-*C.parvum* antibody titre except when considering the 7S uptake by Method D.

TABLE 26

UPTAKE ONTO CBA FIBROSARCOMA CELLS OF IgA AND IgM FROM THE SERUM OF C.PARVUM-TREATED CBA MICE

GOAT ANTISERUM USED	ANTI-MOUSE IgM	ANTI-MOUSE IgA
Mouse serum from Group A[a]	1,391[b]	1,180
Mouse serum from Group B	1,788	1,436
Mouse serum from Group C	1,444	853
Mouse serum from Group D	2,436	1,247

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1×10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1×10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Each result is expressed as counts per 100 sec. and is the mean of triplicates.

Note

1) Taking absorption ratios of 1·3 and over to be significant: serum from Group D gave a significant uptake of IgM when compared to serum from Group A.

2) Absorption ratio for uptake of IgM from Group B serum was 1·29.

3) Antiglobulin Method D used.

TABLE 27 UPTAKE ONTO CBA FIBROSARCOMA CELLS OF IgG SUB-CLASSES FROM THE SERUM OF C.PARVUM-TREATED CBA MICE

GOAT ANTISERUM USED	ANTI-MOUSE IgG_1		ANTI-MOUSE IgG_{2a}		ANTI-MOUSE IgG_{2b}		NORMAL GOAT SERUM	
MOUSE SERUM DIL.	1/8	1/28	1/8	1/28	1/8	1/28	1/8	1/28
Mouse serum[a] from Group A	1,764[b]	1,876	2,709	2,166	1,916	1,853	1,137	1,176
Mouse serum from Group B	2,227	2,131	3,705	3,279	2,221	1,947	1,376	1,264
Mouse serum from Group C	2,432	2,242	2,503	2,149	2,168	1,778	1,479	1,182
Mouse serum from Group D	3,182	2,587	3,532	3,542	3,115	2,428	1,454	1,307

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 105 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Each result is expressed as counts per 300 sec. and is the mean of triplicates.

Note

1) Taking Absorption Ratios of 1·3 and over (compared with serum from Group A) to be significant: serum from Group B gave an uptake of IgG_{2a}; serum from Group C gave an uptake of IgG_1; serum from Group D gave an uptake of IgG_1, IgG_{2a} and IgG_{2b}.

2) The specificity of the goat antisera was shown by using a normal goat serum control.

3) Antiglobulin Method D used.

TABLE 28 EFFECT OF PREINCUBATION[a] WITH AGGREGATED HUMAN IgG (10 µg) ON THE UPTAKE[b] OF IMMUNOGLOBULIN ONTO TUMOUR CELLS

SERUM USED IN ASSAY	CBA FIBROSARCOMA TARGET CELLS				A/HeJ FIBROSARCOMA TARGET CELLS			
	RABBIT ANTI-MOUSE IgG		NORMAL RABBIT SERUM		RABBIT ANTI-MOUSE IgG		NORMAL RABBIT SERUM	
	Preincubation with Agg.H. IgG	NP[c]	Preincubation with Agg.H. IgG	NP	Preincubation with Agg.H. IgG	NP	Preincubation with Agg.H. IgG	NP
Normal mouse serum	1,215	1,291	328	293	1,621	1,546	246	227
Serum from C.parvum (1·4 mg i.p.) treated mice (day 28)	1,994	2,168	273	435	2,395	2,619	322	344
No mouse serum	506	412	232	285	401	415	391	278

a) Preincubation at 37°C for 30 min. Cells then washed twice.

b) Counts per 300 secs.

c) NP - No preincubation.

Note

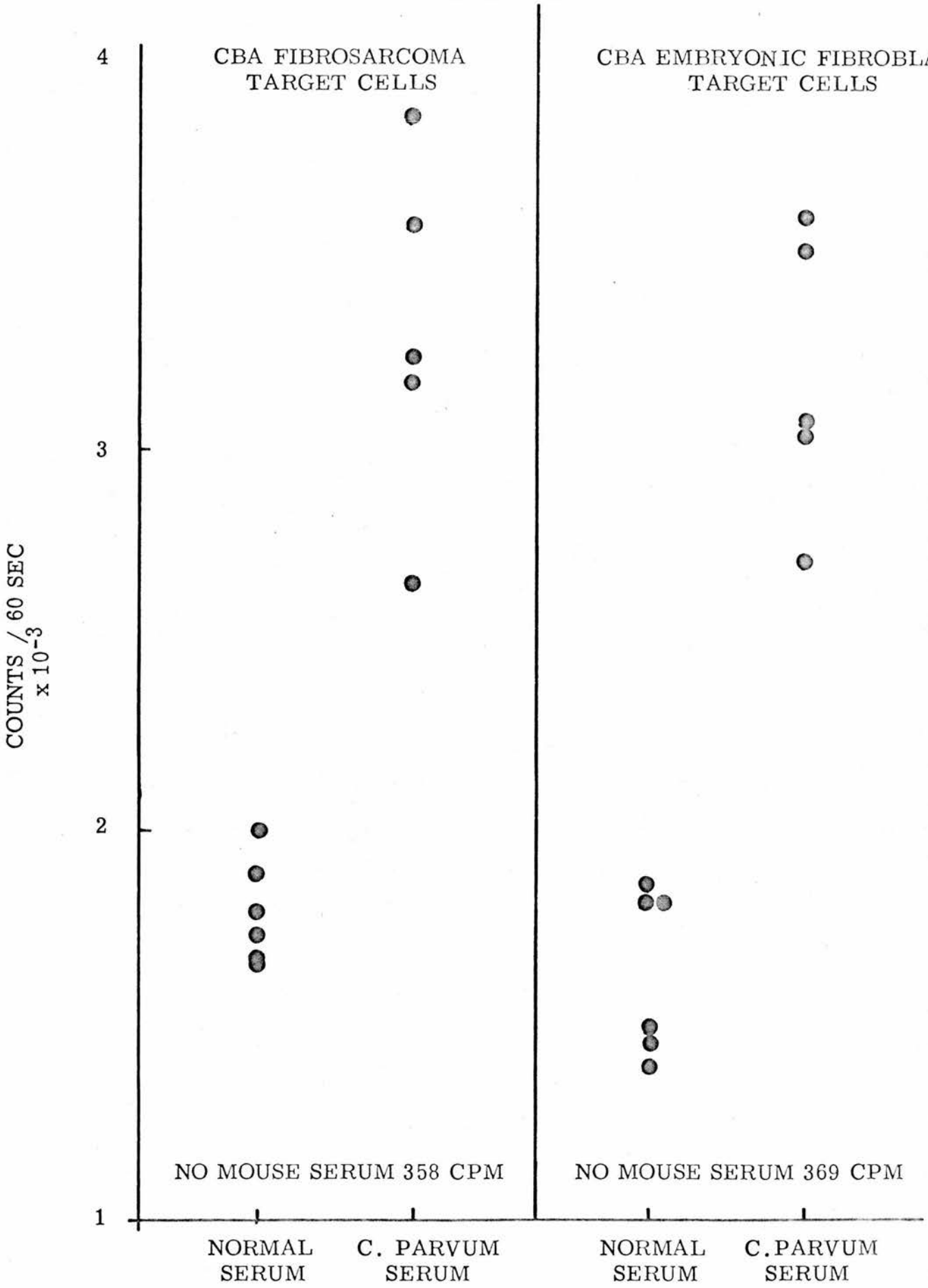
1) CBA tumour cells were also incubated with 40 µg of Agg. Human IgG with the same results as above.

2) Normal serum and serum from C.parvum-treated mice are pools from five individuals.

3) Antiglobulin method B used.

Fig. 28

UPTAKE OF IMMUNOGLOBULIN FROM THE SERUM OF C.PARVUM-TREATED CBA MICE ONTO SYNGENEIC FIBROSARCOMA CELLS AND EMBRYONIC FIBROBLASTS



Note (Fig. 28)

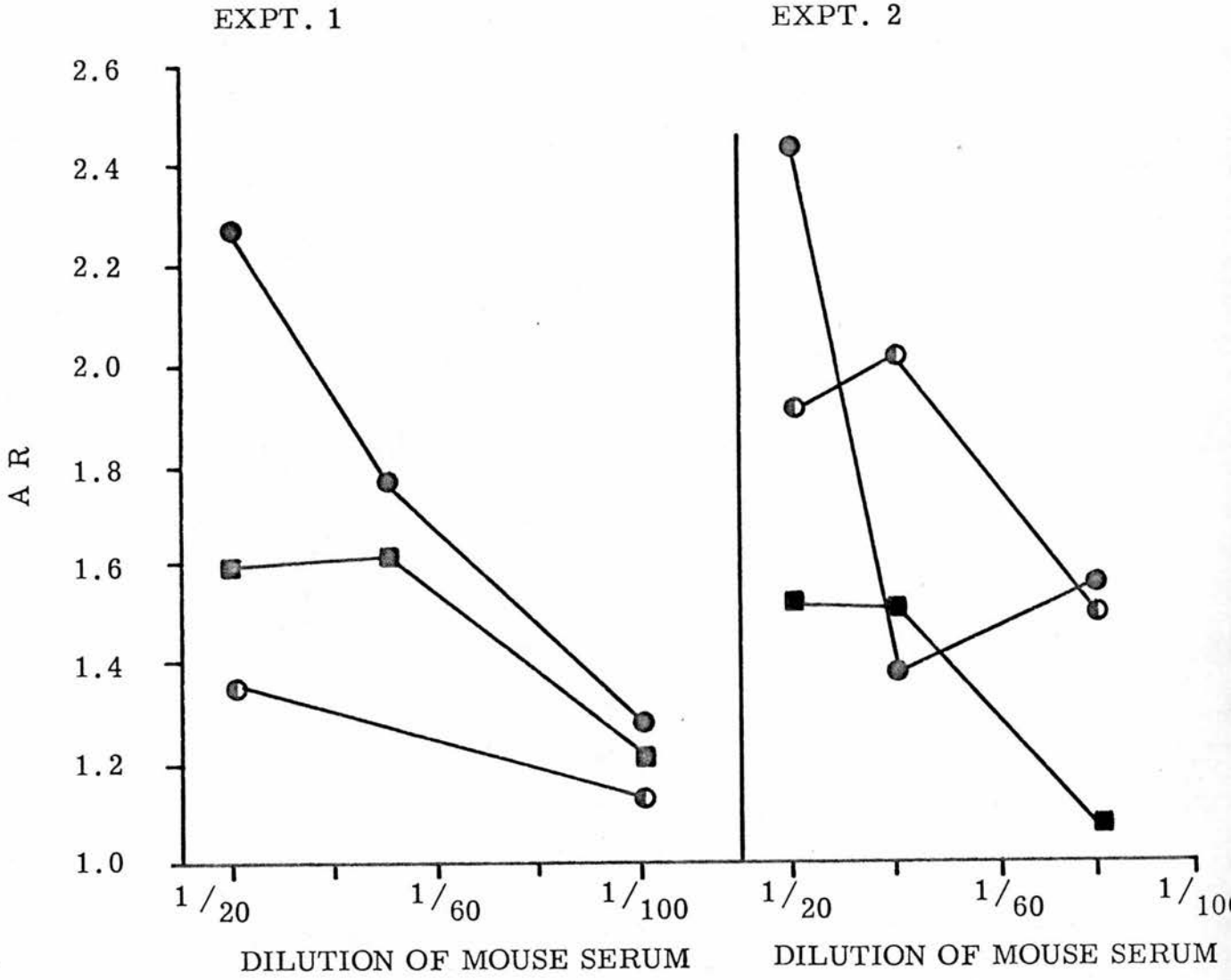
1) C.parvum (1·4 mg) injected i.p. Day 3; mice bled Day 24.

2) Uptake of immunoglobulin from the serum of C.parvum-treated mice was significantly greater than for normal serum ($P < 0{\cdot}001$ for both target cells).

3) Antiglobulin Method B used.

Fig. 29

UPTAKE OF IMMUNOGLOBULIN FROM THE SERUM OF C.PARVUM-TREATED CBA MICE ONTO SYNGENEIC FIBROSARCOMA AND SPLEEN CELLS



TARGET CELLS

- CBA fibrosarcoma (3×10^4)
- CBA spleen (4×10^4)
- CBA spleen (12×10^4)

TARGET CELLS

- CBA fibrosarcoma (3×10^4)
- CBA spleen (4×10^4)
- CBA spleen cells depleted of glass-adherent cells (4×10^4)

$$AR = \frac{\text{CPM WITH SERUM FROM C.PARVUM-TREATED MICE}}{\text{CPM WITH NORMAL SERUM}}$$

Note (Fig. 29)

1) Serum from C.parvum-treated mice (1·4 mg i.p.) was taken 21 days after injection.

2) Uptake of immunoglobulin from the serum of C.parvum-treated mice (expressed as AR) onto CBA fibrosarcoma cells was constant until a dilution of 1/50, whereupon it fell.

3) Uptake of immunoglobulin from the serum of C.parvum-treated mice onto CBA spleen cells was dependent on the serum concentration and the number of target cells. After depletion of glass-adherent cells the uptake onto spleen cells followed a similar pattern to fibrosarcoma cells although the AR was greater.

4) Antiglobulin Method B used.

TABLE 29 SPECIFICITY OF ANTIBODY EVOKED BY C.PARVUM. I

TARGET CELLS	CBA FIBROSARCOMA		CBA EMBRYONIC FIBROBLAST		CBA KIDNEY FIBROBLAST	
MOUSE SERUM FRACTION	19S	7S	19S	7S	19S	7S
Mouse serum[a] from Group A	6,276[b]	5,324	3,220	1,932	2,791	3,312
Mouse serum from Group B	9,290	5,013	4,332	2,200	4,881	4,320
Mouse serum from Group C	4,498	5,840	2,204	2,336	2,416	3,407
Mouse serum from Group D	14,660 (1·58)[c]	9,789 (1·95)	7,626 (1·76)	3,668 (1·67)	8,208 (1·68)	5,533 (1·28)

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Each result is expressed as counts per 60 sec. and is the mean of triplicates.

c) Absorption Ratios for Group D serum (compared with Group B serum) in parentheses. This should give a measure of antibody due to the presence of the tumour, in C.parvum-treated tumour bearing mice.

Note

1) As measured by the Absorption Ratios in parentheses, the 7S antibody response exhibited a degree of specificity for CBA fibrosarcoma cells whereas the 19S response did not.

2) Antiglobulin Method D used.

TABLE 30 SPECIFICITY OF ANTIBODY EVOKED BY C.PARVUM. II

TARGET CELLS	CBA FIBROSARCOMA	CBA FIBROSARCOMA PRETREATED WITH AGGREGATED HUMAN IgG (45 µg)	CBA EMBRYONIC FIBROBLAST	A/HeJ FIBROSARCOMA
Mouse serum [a] from Group A (7S fraction)	2,315[b]	1,950	2,138	1,671
Mouse serum from Group B (7S fraction)	2,272	2,132	2,368	1,915
Mouse serum from Group C (7S fraction)	2,412	2,343	2,291	1,976
Mouse serum from Group D (7S fraction)	4,758 (2·10)[c]	4,445 (2·08)	3,812 (1·60)	3,415 (1·78)

a) Group A - normal CBA controls; Group B - 1½ mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Each result is expressed as counts per 60 sec. and is the mean of triplicates.

c) Absorption Ratios for Group D serum (compared with Group B serum) in parentheses.

Note

1) Pretreatment with aggregated human IgG failed to block the uptake of 7S immunoglobulin onto CBA fibrosarcoma cells.

2) Uptake onto CBA fibrosarcoma cells was higher than for CBA embryo cells or A/HeJ fibrosarcoma cells

3) Antiglobulin Method D used.

TABLE 31 SPECIFICITY OF ANTIBODY EVOKED BY C.PARVUM. III

TARGET CELLS	CBA FIBROSARCOMA		CBA SPLEEN CELLS		CBA SPLEEN CELLS PREINCUBATED WITH AGGREGATED HUMAN IgG (40 µg)	
MOUSE SERUM FRACTION	19S	7S	19S	7S	19S	7S
Mouse serum[a] from Group A	3,698 [b]	1,905	2,884	2,100	3,052	2,218
Mouse serum from Group B	3,726	2,207	2,763	2,219	2,891	2,435
Mouse serum from Group C	2,997	2,220	2,320	2,160	2,679	2,534
Mouse serum from Group D	4,833 (1·30)[c]	4,637 (2·12)	4,226 (1·53)	4,425 (1·99)	4,160 (1·44)	3,536 (1·47)

a) Group A - normal CBA controls; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

b) Each result is expressed as counts per 60 sec. and is the mean of triplicates.

c) Absorption Ratios for Group D serum (compared with Group B serum) in parentheses.

Note

1) Pretreatment with aggregated human IgG partly inhibited the uptake of 7S immunoglobulin onto syngeneic spleen cells, whereas it only marginally affected the 19S uptake.

2) Antiglobulin Method D used.

TABLE 32 SUMMARY OF SPECIFICITY DATA USING NORMAL AND MALIGNANT TARGET CELLS

COUNTS PER UNIT TIME WITH 7S FRACTION OF SERUM D [a] / COUNTS PER UNIT TIME WITH 7S FRACTION OF SERUM B [a]	TARGET CELLS	NO. OF TIMES TESTED
1·0 - 1·5	CBA kidney	1
	CBA spleen pretreated with aggregated IgG	1
1·5 - 1·8	CBA embryo	2
	A/HeJ fibrosarcoma	1
> 1·9	CBA fibrosarcoma	3

a) Serum B - 1·h mg C.parvum i.p. Day 3; Serum D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0, 1·h mg C.parvum i.p. Day 3; mice bled Day 2h.

Note

1) All target cells, with the exception of spleen cells, were in vitro cultured cell lines.

2) Only CBA fibrosarcoma cells gave a consistently high Absorption Ratio.

3) The Absorption Ratios for the 19S fractions are ranked as follows: CBA embryo > CBA kidney > CBA fibrosarcoma.

TABLE 33 THE EFFECT OF ABSORPTION WITH C.PARVUM, TUMOUR AND SPLEEN ON THE ANTIBODY ACTIVITY OF MOUSE SERUM POOLS

POOL	DONOR TREATMENT(a)	ABSORPTION(b) WITH	TUMOUR ANTIBODIES counts/min.		C.PARVUM ANTIBODIES $\log_2$ titre	
			BEFORE	AFTER	BEFORE	AFTER
2C	Tumour (day 0)	Tumour	2574	2535	2·8	2·8
		C.parvum	2574	1948	2·8	<2·3
		Spleen	2574	2217	2·8	3·3
2D	Tumour (day 0), C.parvum (day 3)	Tumour	3314	2255	8·8	8·1
		C.parvum	3314	2591	8·8	2·8
		Spleen	3314	3208	8·1	8·6
3C	Tumour (day 0)	Tumour	4725	4762	<3·4	<3·4
		C.parvum	4725	4111	<3·3	<3·3
		Spleen	4725	4301	<3·4	<3·4
3D	Tumour (day 0), C.parvum (day 3)	Tumour	7901	5166	8·9	8·2
		C.parvum	7901	4800	8·3	4·7
		Spleen	7901	5724	8·9	9·2

a) 1·4 mg of C.parvum CN 6134 administered i.p. 3 days after s.c. transplantation of 1 x 10^5 viable CBA fibrosarcoma cells.

b) 1 ml of serum (dil x 10) absorbed x 3 with C.parvum or syngeneic tumour or spleen cells and antibodies determined simultaneously in absorbed and unabsorbed serum.

Note

1) Absorption with tumour reduced the antitumour antibody levels in C.parvum-treated mice but had little effect on C.parvum antibody titres.

2) Absorption with C.parvum reduced the levels of antitumour antibodies and C.parvum antibodies.

3) Spleen was less effective than C.parvum or tumour at absorbing out antitumour antibodies.

4) Antiglobulin Method B was used to examine Pool 2, and Method D for Pool 3.

binding to CBA fibrosarcoma cells *in vitro*, produced after *C.parvum* administration, could be absorbed out by CBA fibrosarcoma cells, *C.parvum*, and to a lesser extent by CBA spleen cells (Table 33). It is of interest that absorption with CBA fibrosarcoma cells, of serum from CBA fibrosarcoma bearing mice, did not result in a decrease in immunoglobulin binding to CBA fibrosarcoma cells *in vitro*. However, absorption with CBA spleen cells did. This could be due to Fc-receptor bearing cells in the spleen cell population, and illustrates the necessity for adequate control sera. It should be noted that absorption with either type of cell did not reduce the anti-*C.parvum* antibody titre. Only *C.parvum* was effective in this respect.

3. USE OF ANTIGLOBULIN ASSAY TO DETECT ANTI-TUMOUR ANTIBODY ELICITED WITHOUT THE INTERVENTION OF C.PARVUM

On the basis of reports from Thomson *et al.* (154) and Burdick *et al.* (45) that, respectively, excision of the tumour burden and hyper-immunisation with tumour cells elicited anti-tumour antibody in a syngeneic system, these methods were tried in our tumour model.

a) Production of anti-tumour antibody by excision of tumour burden

CBA mice were injected on the upper aspect of the foot with 1 x 10^5 syngeneic fibrosarcoma cells. 14 Days later the tumour bearing leg was amputated and the mice exsanguinated at intervals. Depending on the experiment, controls were either untreated mice, sham-operated mice, or mice which still had a tumour burden. Three separate experiments are recorded, all of which use antiglobulin method B to detect anti-tumour antibody.

Using pooled serum from bleeds at various intervals it appeared from Fig. 30 that serum from animals with their tumour burden removed had higher levels of anti-tumour antibody than mice retaining their tumour burden, or untreated mice. However, when this experiment was repeated using the serum from each individual mouse the picture was not so clear in that the anti-tumour antibody levels in serum from mice with their tumour burden removed were similar to controls (Fig. 31).

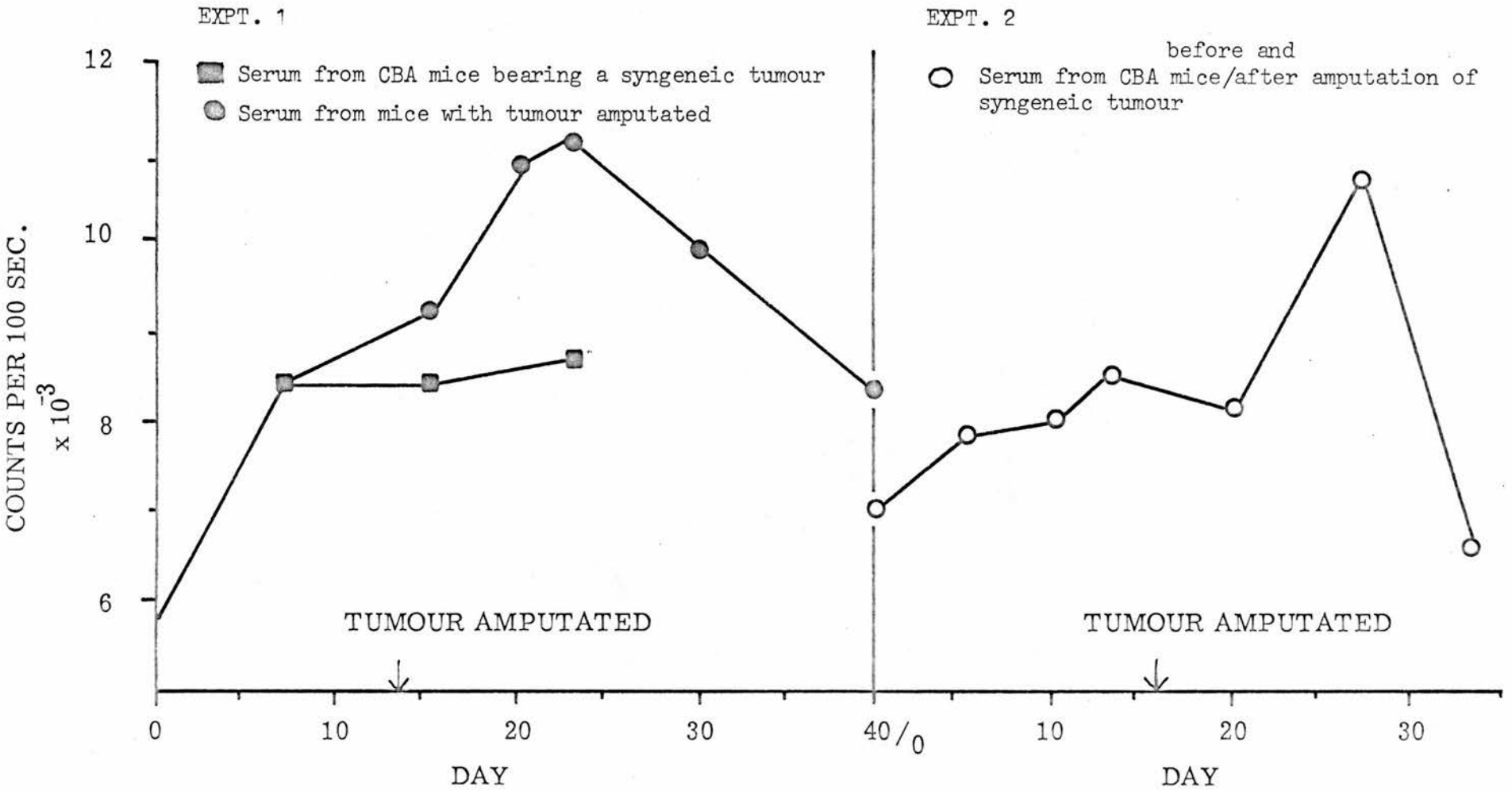
b) Production of anti-tumour antibody by hyperimmunisation with syngeneic tumour cells

In the first experiment CBA mice were injected with 10^6 X-irradiated (22,000 RAD) syngeneic fibrosarcoma cells s.c. to render them immune to a subsequent challenge of live tumour cells. At two week intervals they were challenged s.c. with increasing doses of live CBA fibrosarcoma cells starting with 10^2. The mice were bled from the eye (and the serum pooled) at various intervals after challenges of both 1×10^5 and 4×10^5 tumour cells (Table 34). Following the final challenge (4×10^5 cells) some mice had small nodules at the site of injection; these had regressed by day 8.

In a second experiment the mice were made immune to a final challenge of 4×10^6 CBA fibrosarcoma cells and were exsanguinated 21 days later. Of the experimental group of 10 mice challenged with 4×10^6 tumour cells, 4 grew tumours. Thus, in Fig. 32 there are three experimental groups namely: untreated mice; mice immune to 4×10^6 CBA fibrosarcoma cells; and mice which succumbed to a challenge of 4×10^6 cells. Individual sera from the three groups were examined for antibody against CBA fibrosarcoma cells and CBA embryonic

Fig. 30 APPEARANCE OF IMMUNOGLOBULIN BINDING TO CBA FIBROSARCOMA CELLS AFTER TUMOUR AMPUTATION (EXPTS. 1 AND 2)

(EACH POINT REPRESENTS A SERUM POOL FROM AT LEAST FOUR MICE)



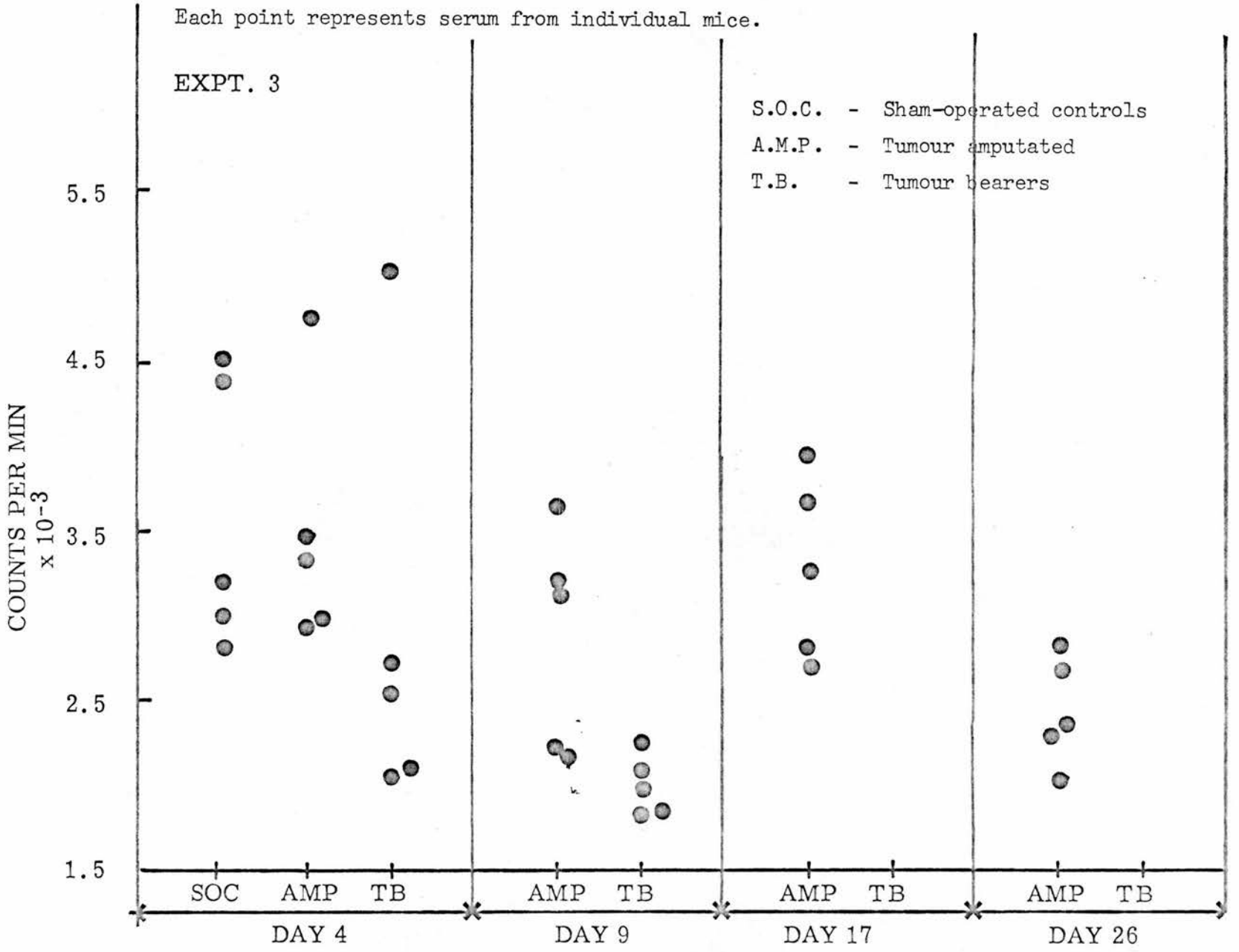
Note (Fig. 30)

1) CBA fibrosarcoma cells (1×10^5) injected into foot of syngeneic mice Day 0; foot amputated from knee at points shown when tumour was palpable; mice bled on days shown and sera pooled.

2) In Experiments 1 and 2 immunoglobulin binding to tumour cells was produced transiently after amputation.

3) Antiglobulin Method B used.

Fig. 31 APPEARANCE OF IMMUNOGLOBULIN BINDING TO CBA FIBROSARCOMA CELLS AFTER TUMOUR AMPUTATION ON DAY 0 (EXPT. 3)



Note (Fig. 31)

1) CBA fibrosarcoma cells (1×10^5) injected into foot of syngeneic mice and 14 days later the tumour bearing foot amputated at the knee- Day 0; mice bled on the days shown and sera tested individually.

2) In Experiment 3 no significant increase in immunoglobulin binding to tumour cells was seen after amputation.

3) Antiglobulin Method B used.

In retrospect, it might be pointed out that although the level of immunoglobulin binding to CBA fibrosarcoma cells in vitro, in the serum of mice deprived of their tumours, was no greater than for sham-operated controls, it was greater than for tumour bearing mice.

TABLE 34

APPEARANCE OF ANTI-TUMOUR ANTIBODY IN CBA MICE AFTER IMMUNISATION WITH SYNGENEIC TUMOUR CELLS

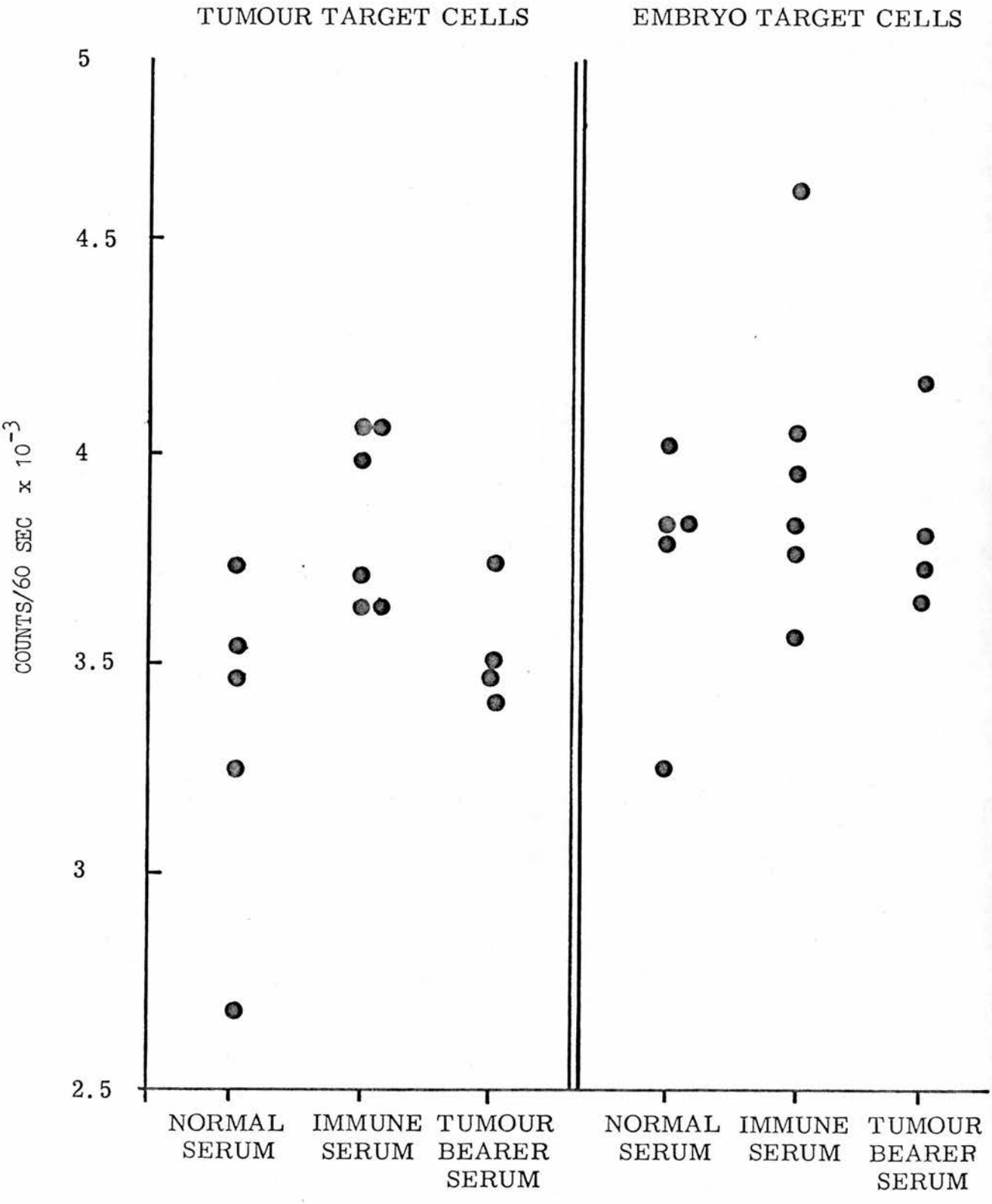
DAY AFTER CHALLENGE WHEN BLED FROM ORBITAL SINUS	A.R.[a] OF SERUM FROM MICE IMMUNE TO A CHALLENGE OF	
	1×10^5 CBA FIBROSARCOMA CELLS	4×10^5 CBA FIBROSARCOMA CELLS
8	NT [b]	1·83
13	1·20	NT
25	NT	2·12
53	NT	1·53

a) Antiglobulin method B used. Target cells were CBA fibrosarcoma

$$AR = \frac{\text{COUNTS PER UNIT TIME WITH SERUM FROM HYPERIMMUNE MICE}}{\text{COUNTS PER UNIT TIME WITH SERUM FROM NORMAL MICE}}$$

b) NT - not tested

Fig.32 UPTAKE OF IMMUNOGLOBULIN FROM THE SERUM OF MICE IMMUNE TO A SYNGENEIC CBA FIBROSARCOMA (USING ANTIGLOBULIN METHOD B)



Note (Fig. 32)

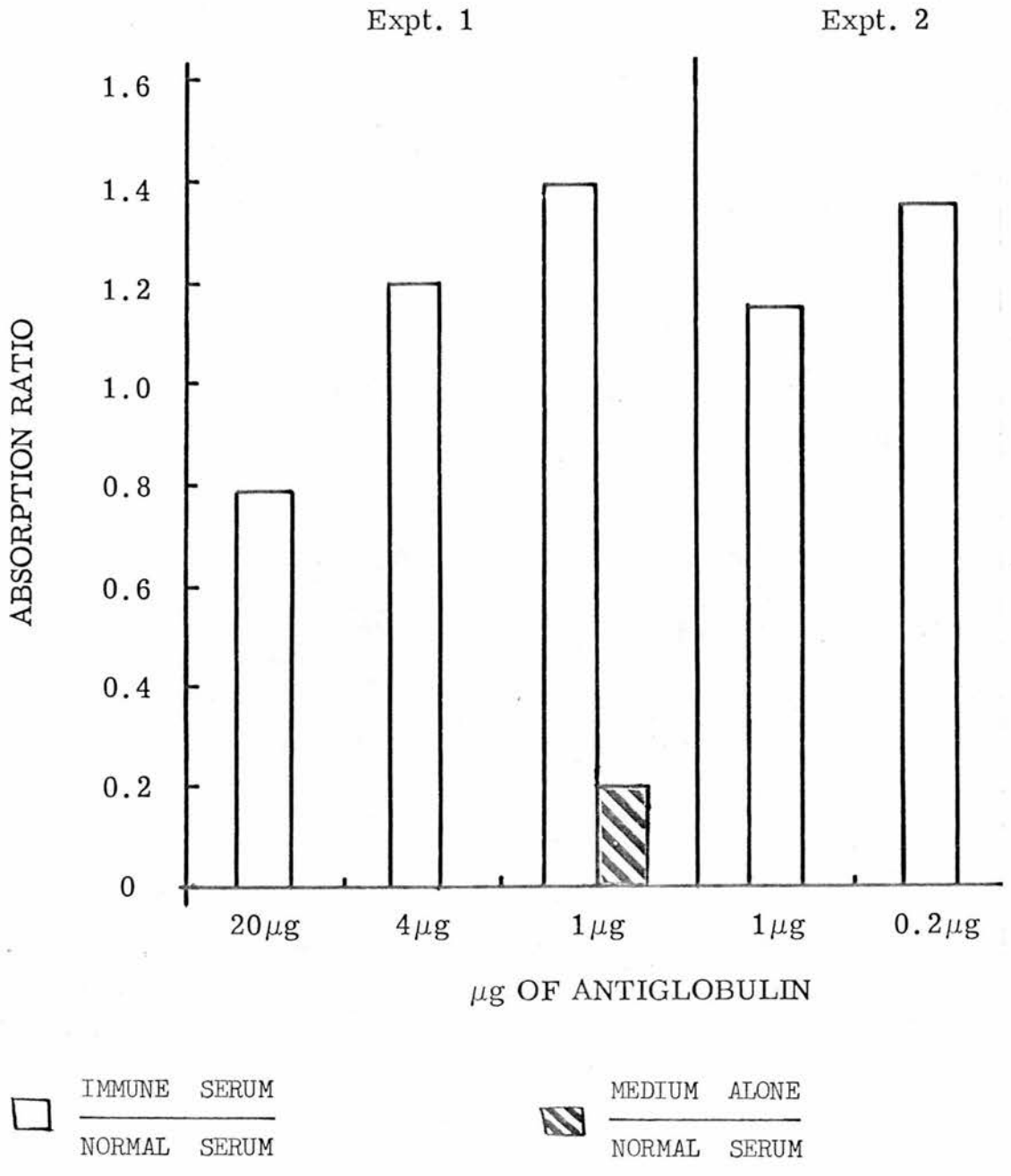
1) CBA mice were injected with 10^6 X-irradiated syngeneic fibrosarcoma cells then at two week intervals they were challenged with increasing doses of live pronase digested tumour cells starting at 1×10^3 and ending at 4×10^6. After the last challenge 4 mice grew tumours and 6 remained tumour free.

2) Using CBA fibrosarcoma cells as targets in the antiglobulin assay the uptake of serum from tumour immune mice was significantly greater than normal serum ($P < 0{\cdot}025$). However the uptake from the serum of tumour bearing mice was not significantly greater than normal serum.

Using CBA embryo target cells there were no significant differences between serum from normal, tumour bearing and tumour immune mice.

Fig. 33

UPTAKE OF IMMUNOGLOBULIN FROM THE SERUM OF MICE IMMUNE TO A SYNGENEIC CBA FIBROSARCOMA (USING ANTIGLOBULIN METHOD C)



Note

1) To obtain tumour immune serum CBA mice were injected with 10^6 X-irradiated syngeneic fibrosarcoma cells then at two week intervals they were challenged with increasing doses of live pronase digested tumour cells starting at 1×10^3 and ending at 4×10^6. Normal serum was from age matched controls.

2) In two experiments Absorption Ratios of 1·4 and 1·35 were obtained for immune serum.

3) Immunoglobulin from normal mouse serum was taken up onto tumour cells compared with a medium only control.

fibroblasts using antiglobulin method B (Fig. 32), and in addition the first two groups were tested using antiglobulin method C against CBA fibrosarcoma target cells only (Fig. 33).

The above results indicate that both hyperimmunisation with syngeneic tumour cells and amputation of a syngeneic tumour elicit anti-tumour antibody detectable in the antiglobulin assay. However, results from amputated mice were rather questionable. This is because higher levels of circulating anti-tumour antibody were observed in amputated mice than in mice with an intact tumour burden, but the levels were not higher than in sham-operated controls. Furthermore, when anti-tumour antibody was detected it appeared transiently.

Results from hyperimmunised mice were again quantitatively rather variable although anti-tumour antibody was always detectable after immunisation. Furthermore, Table 34 indicates that a threshold dose of cells was necessary before anti-tumour antibody could be detected. The specificity of anti-tumour antibody produced by hyperimmunisation was also examined. Fig. 32 shows that only tumour-free immune mice had significant levels of antibody directed against syngeneic fibrosarcoma cells, whereas mice with a tumour burden had not. Furthermore, this antibody was not directed towards embryonic antigens as the uptake of immunoglobulin from immune serum was not significantly different to normal serum when tested against embryonic fibroblasts.

4. ESTIMATION OF SERUM IMMUNOGLOBULIN CLASS AND SUB-CLASS LEVELS AFTER C.PARVUM ADMINISTRATION

These studies were carried out on the same serum samples as for section 2 and both sets of results are meant to complement each other.

Rather than slavishly reproduce parts of section 2 here the experimental protocols are given with the relevant Figures and Tables, and only a summary of the results is given here.

a) Effect of route of administration and dose of C.parvum on serum immunoglobulin class and sub-class levels

In addition to the inhibition of tumour growth, the i.p. administration of C.parvum (group F) resulted in a significant increase of all immunoglobulin levels (except IgM) in the sera of tumour bearing mice (Fig. 34). In contrast, C.parvum given s.c. (Group E) failed to influence tumour growth and had little, if any, effect on circulating immunoglobulin levels. The i.p. administration of C.parvum to normal mice (group C) resulted in a significant increase in both IgG_{2a} and IgG_{2b} levels, but the s.c. injection was again without effect (group B).

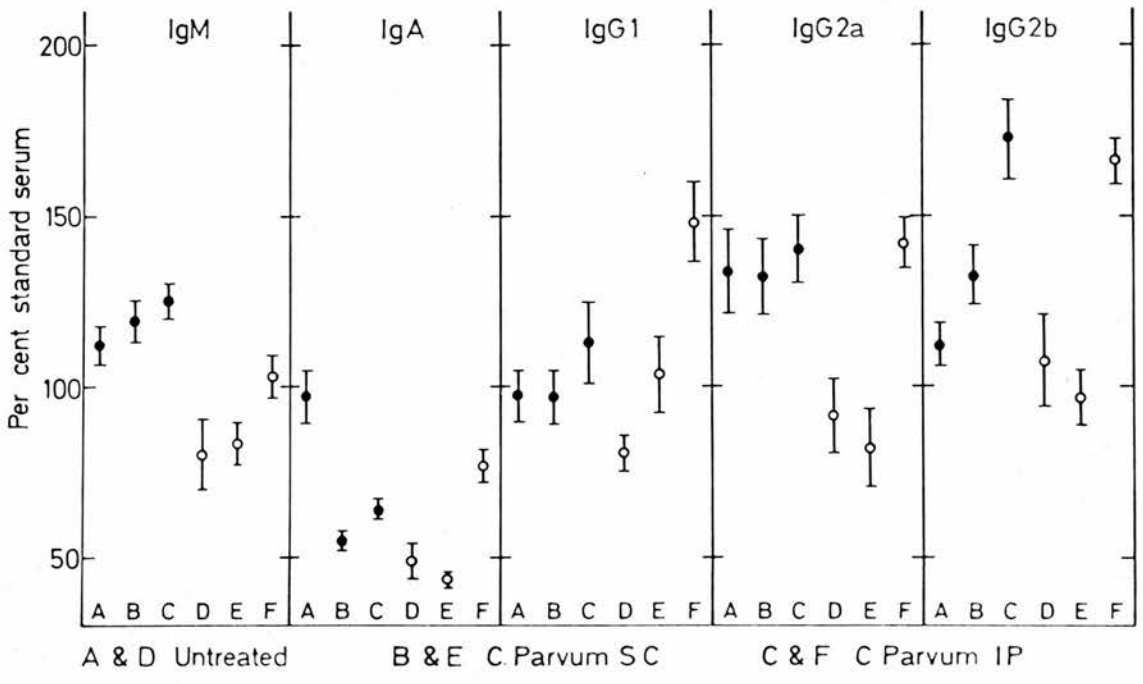
The highest doses of C.parvum elicited the greatest increase in immunoglobulin levels in normal mice; IgM and IgG_{2b} levels being significantly raised after injection of 1·4 mg of C.parvum (Fig. 35).

b) Cellular basis for the changes in serum immunoglobulin class and sub-class levels, after C.parvum administration

The tumour-inhibitory effect of C.parvum was abrogated by treatment with gold salts. Furthermore, the levels of all immunoglobulins (except IgA) were significantly higher in tumour bearing mice given the combined C.parvum-gold salt treatment (group D), than in untreated tumour bearing controls (group A). However, the IgM, IgG_{2a} and IgG_{2b} levels in Group D were significantly lower than in mice receiving C.parvum alone (group B) (Fig. 36).

Fig. 34

EFFECT OF ROUTE OF C.PARVUM INJECTION ON SERUM IMMUNOGLOBULIN LEVELS IN NORMAL (●) AND TUMOUR BEARING CBA MICE (O)



● NORMAL O TUMOUR BEARING

Note

1) Tumour (1×10^5 cells) s.c. Day 0; C.parvum (1·4 mg) i.p. or s.c. on leg opposite to tumour, Day 3; mice bled Day 24.

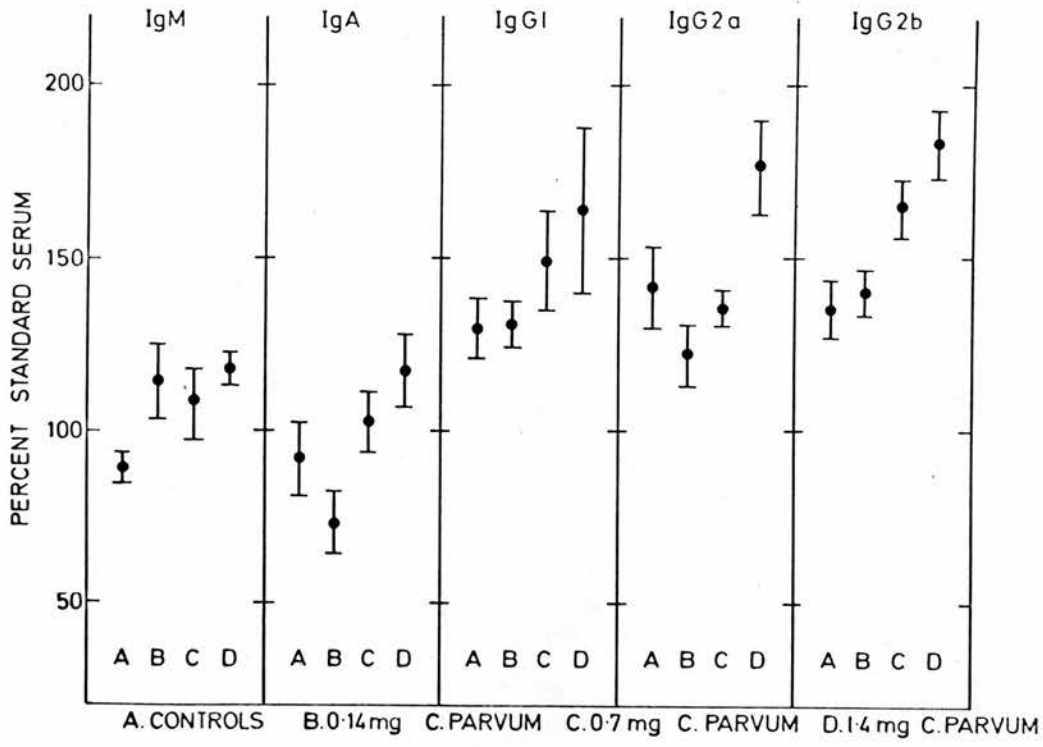
2) Immunoglobulin levels not significantly increased by s.c. injection of C.parvum in normal or tumour bearing mice.

3) IgG_{2a} ($P < 0{\cdot}02$) and IgG_{2b} ($P < 0{\cdot}001$) levels significantly raised in normal mice after C.parvum injection i.p.

4) IgG_{2a} ($P < 0{\cdot}001$), IgG_{2b} ($P < 0{\cdot}005$), IgG_1 ($P < 0{\cdot}001$), IgA ($P < 0{\cdot}005$) levels significantly raised in tumour bearing mice after C.parvum injection i.p.

5) Only i.p. injection of C.parvum inhibited tumour growth ($P < 0{\cdot}05$).

Fig. 35 EFFECT OF DIFFERENT DOSES OF C.PARVUM ON IMMUNOGLOBULIN LEVELS IN CBA MICE



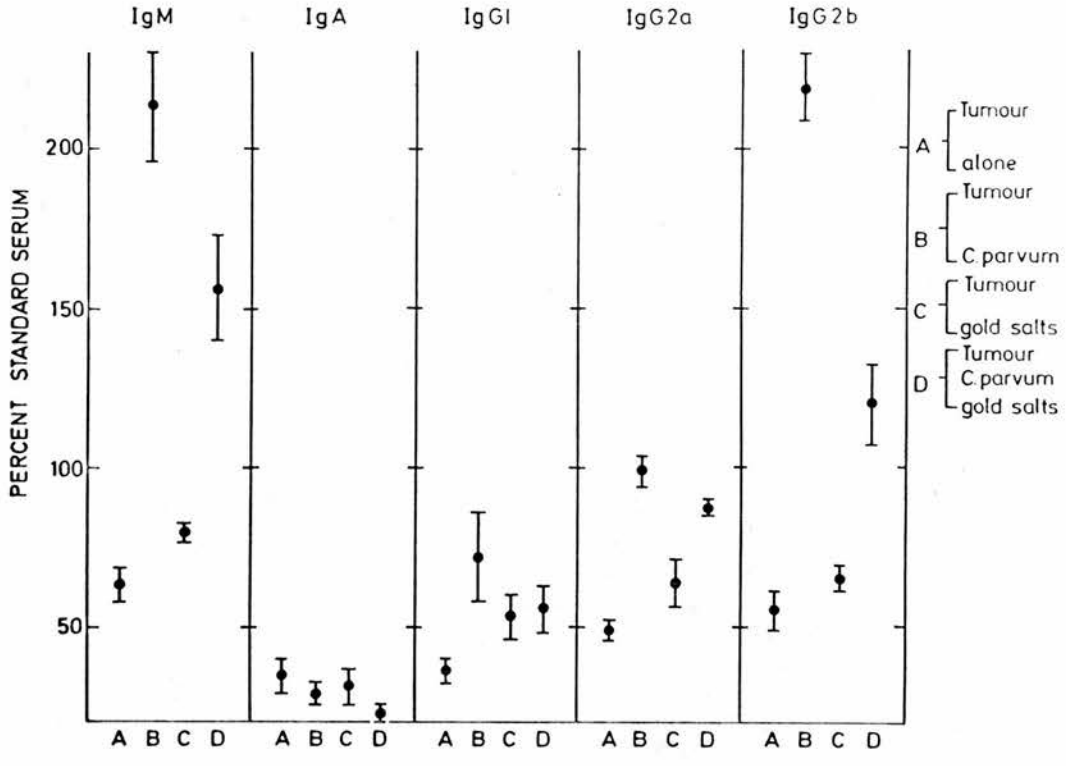
Note

1) (all doses) C.parvum/injected i.p. Day 0; mice bled Day 21.

2) IgM ($P < 0{\cdot}001$) and IgG_{2b} ($P < 0{\cdot}005$) significantly raised after injection of 1·4 mg of C.parvum i.p.

Fig. 36

EFFECT OF GOLD SALTS ON IMMUNOGLOBULIN LEVELS IN C.PARVUM-TREATED TUMOUR BEARING CBA MICE



Note

1) Tumour (1×10^4 cells) injected s.c. Day 0; C.parvum (1·4 mg) injected i.p. Day 3; sodium aurothiomalate was administered by eight i.p. injections on alternate days starting Day 2; mice bled Day 24.

2) IgM ($P < 0{\cdot}05$), IgG_{2a} ($P < 0{\cdot}01$), IgG_{2b} ($P < 0{\cdot}001$) levels significantly lower in mice receiving the combined C.parvum-gold salt therapy than in mice receiving C.parvum alone.

3) The administration of C.parvum (Group B) significantly inhibited tumour growth ($P < 0{\cdot}01$) but this effect could be overcome by administration of gold salts (Group D).

TABLE 35

A COMPARISON OF THE EFFECTS OF C.PARVUM AND P.FREUDENREICHII ON IMMUNOGLOBULIN LEVELS AND TUMOUR GROWTH

GROUP	TUMOUR	ADJUVANT (a)	IgM	IgA	IgG_1	IgG_{2b}	TUMOUR DIAMETER (b)
			mg/dl (b)				
A	No	No treatment	45 (43-48)	103 (94-112)	288 (259-319)	101 (92-110)	-
B	No	C.parvum strain CN 6134 (1·4 mg)	43 (40-47)	115 (106-124)	240 (206-279)	131* (119-143)	-
C	No	Propionibacterium freudenreichii strain 10470 (1·4 mg)	90*** (c) (81-100)	93 (84-103)	368 (319-423)	253*** (230-280)	-
D	No	C.parvum strain 10387 (0·7 mg)	119*** (112-125)	68** (64-73)	359 (336-384)	224*** (212-236)	-
E	Yes	No treatment	25*** (23-28)	65** (58-73)	253 (234-274)	56** (49-63)	16·3 (15·5-17·2)
F	Yes	C.parvum strain CN 6134 (1·4 mg)	32 (30-35)	95 (83-110)	297 (274-323)	89*** (81-98)	9·2** (7·9-10·3)
G	Yes	Propionibacterium freudenreichii strain 10470 (1·4 mg)	70*** (65-76)	61 (53-70)	340 (299-385)	114** (100-130)	14·6 (13·3-16·0)
H	Yes	C.parvum strain 10387 (0·7 mg)	92*** (84-100)	62 (57-67)	421*** (398-446)	132*** (121-144)	9·0*** (8·1-9·9)

a) Adjuvant administered i.p. 3 days after s.c. transplantation of 1 x 10^5 viable CBA fibrosarcoma cells.
b) All values observed 21 days after adjuvant administration ie. 24 days after tumour transplantation.
c) The significance of effects observed was determined by comparing Gps. B to E with A, and Gps. F to H with E.
In all tables * denotes $P \leq 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P \leq 0{\cdot}001$. All other values were not significantly different.

Note
1) The most marked effects were on IgG_{2b} (all strains) and IgM levels (strains 10470 and 10387);
2) strains CN 6134 and 10387 significantly inhibited tumour growth.

TABLE 36

A COMPARISON OF THE EFFECTS OF C.PARVUM AND OTHER ADJUVANTS ON IMMUNOGLOBULIN LEVELS AND TUMOUR GROWTH

GROUP	TUMOUR	ADJUVANT (a)	IgM	IgA	IgG_1	IgG_{2a}	IgG_{2b}	TUMOUR DIAMETER (b)
			mg/dl (b)					
A	No	No	43 (40-46)	181 (169-192)	353 (331-376)	415 (403-428)	112 (106-118)	-
B	No	C.parvum CN 6134 (0·7 mg)	48 (45-50)	188 (178-199)	352 (324-383)	579*** (c) (548-613)	167*** (157-179)	-
C	No	B.C.G. (9×10^5)	39 (38-41)	156 (142-171)	295 (262-333)	494** (476-512)	113 (110-116)	-
D	No	B.pertussis (4×10^9)	55* (53-58)	117*** (107-130)	272 (240-308)	658*** (589-734)	152*** (144-160)	-
E	Yes	No	21*** (18-23)	79*** (69-91)	128*** (113-145)	137*** (109-173)	38*** (34-42)	18·4 (17·6-19·3)
F	Yes	C.parvum CN 6134 (0·7 mg)	40*** (34-48)	132** (125-139)	218** (176-271)	549*** (511-591)	132*** (125-139)	11·3*** (10·6-12·0)
G	Yes	B.C.G. (9×10^5)	24 (23-25)	81 (74-89)	180* (169-192)	143 (105-195)	65** (60-70)	17·2 (16·7-17·8)
H	Yes	B.pertussis (4×10^9)	29* (26-32)	67 (58-78)	174* (164-185)	382*** (349-418)	105*** (100-110)	15·5* (14·7-16·4)

a) Adjuvant administered i.p. 3 days after s.c. transplantation of 1×10^5 viable CBA fibrosarcoma cells.
b) All values observed 21 days after adjuvant administration. Values in Gp. B to E compared with A and Gp. F to H with E.
c) * denotes $P \leq 0{\cdot}05$, ** denotes $P \leq 0{\cdot}01$, *** denotes $P < 0{\cdot}001$. All other values were not significantly different.

Note

1) C.parvum CN 6134 and B.pertussis protocols exerted a more marked effect than the B.C.G. protocol on Ig levels (especially IgG_{2a} and IgG_{2b}) in both normal and tumour bearing mice;
2) C.parvum CN 6134 alone caused appreciable inhibition of tumour growth.

The anti-tumour effect of C.parvum was apparent in T-cell-deficient mice, and the administration of C.parvum to thymectomised tumour bearing mice resulted in an increase in the levels of all serum immunoglobulins except IgG_1 (Table 22). In addition, "nude" mice exhibited elevated serum immunoglobulin levels after C.parvum administration (Table 23).

c) Effect of different strains of C.parvum, in normal and tumour bearing mice, on serum immunoglobulin class and sub-class levels

All the adjuvants induced a significant increase in serum IgG_{2b} levels in both normal and tumour bearing mice, whereas only C.parvum 10387 (groups D and H) and Propionibacterium freudenreichii (groups C and G) elicited significant increases in IgM levels. Only the C.parvum strains (CN 6134 and 10387) caused significant inhibition of tumour growth (Table 35).

d) Effect of different adjuvants, in normal and tumour bearing mice, on serum immunoglobulin class and sub-class levels

The C.parvum (group B) and Bordetella pertussis (group D) treatments used resulted in a marked increase in IgG_{2a} and IgG_{2b} levels in normal mice. In addition, they also caused a significant increase in the levels of all immunoglobulins (except IgA) in tumour bearing mice (groups F and H). The BCG protocol in general elicited small increases, for example IgG_{2a} levels in normal mice (group C) and IgG_1 and IgG_{2b} levels in tumour bearing mice (group G). Only the C.parvum protocol produced substantial inhibition of tumour growth (Table 36).

5. DETECTION OF ANTIBODY CYTOSTATIC FOR CBA FIBROSARCOMA CELLS IN THE SERUM OF C.PARVUM-TREATED MICE

To investigate whether the results from the antiglobulin assays were

due to a genuine antigen-antibody interaction, an assay which utilised this interaction was necessary. This requirement was fulfilled by the complement-dependent assay for cytotoxic antibody as described by Le Mevel and Wells (155). The technique in fact measures cytostatic antibody as the ^{125}I-iododeoxyuridine is added after the target cells have been incubated with the test serum. A measure is thereby obtained of the number of cells remaining that are capable of division.

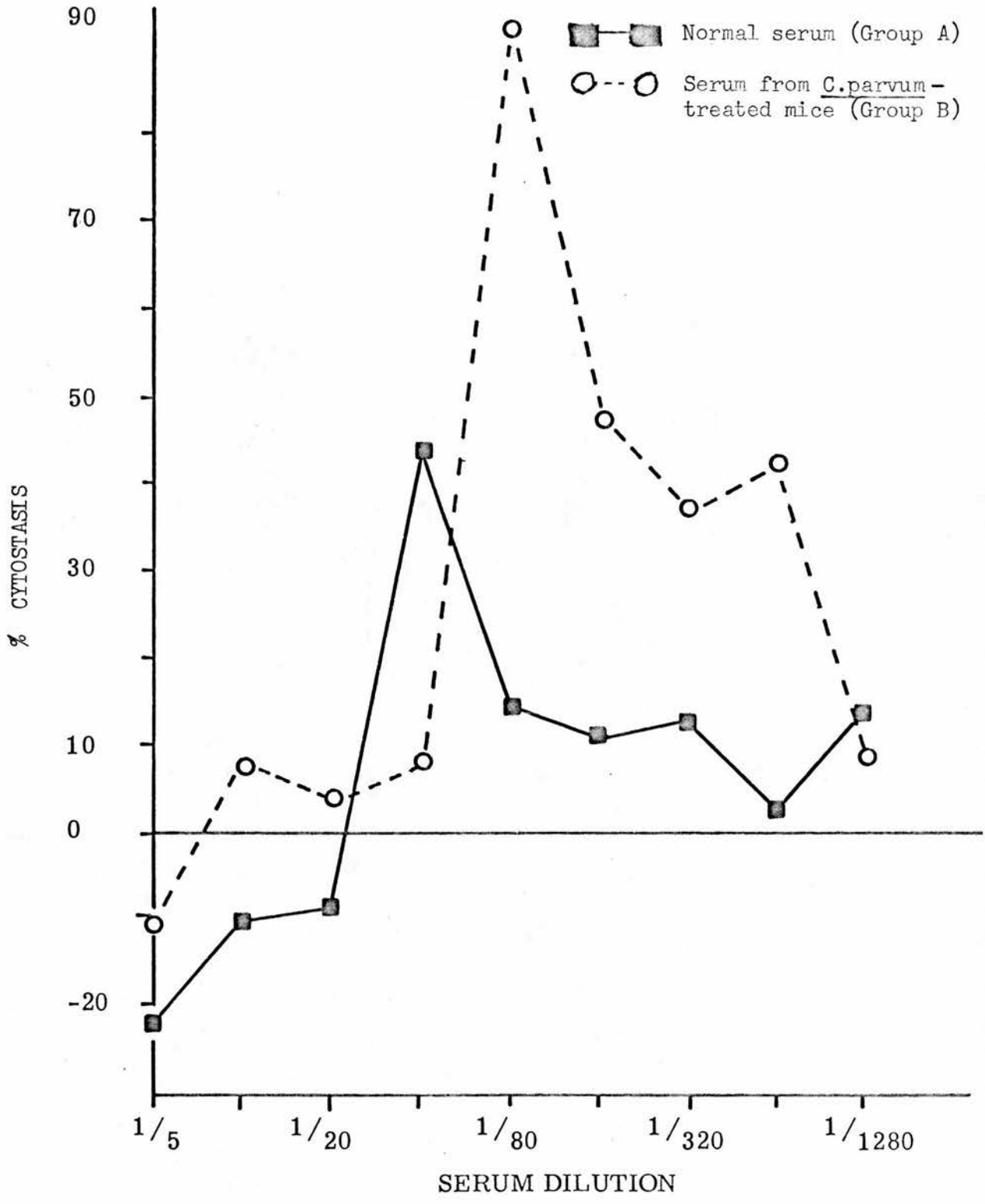
These experiments investigated whether the injection of C.parvum into normal mice or tumour bearing mice elicited higher levels of cytostatic antibody than the relevant controls (ie. serum from normal and tumour bearing mice respectively). The system was the syngeneic CBA model used previously and the experimental sera examined were from Group A - normal CBA mice; group B - CBA mice treated with C.parvum; group C - CBA mice bearing a syngeneic fibrosarcoma; group D - CBA mice bearing a syngeneic fibrosarcoma treated with C.parvum.

It can be seen from Figs. 37 and 38 that the controls (normal and tumour bearer mouse serum) could exert a cytostatic effect. When this was the case the results were expressed as follows:-

$$\% \text{ CYTOSTASIS} = \left[1 - \frac{\text{COUNTS PER UNIT TIME WITH SERUM FROM GROUP A, B, C OR D + COMPLEMENT}}{\text{COUNTS PER UNIT TIME WITH MEDIUM + COMPLEMENT}} \right] \times 100$$

When the control serum was inactive (less than 30% cytostasis) the results were expressed as a percentage of this control (Fig. 39). For groups C and D this could be expressed as follows:-

Fig.37 CYTOSTATIC ACTIVITY OF SERUM FROM NORMAL AND C.PARVUM-TREATED CBA MICE AGAINST SYNGENEIC FIBROSARCOMA CELLS



Note (Fig. 37)

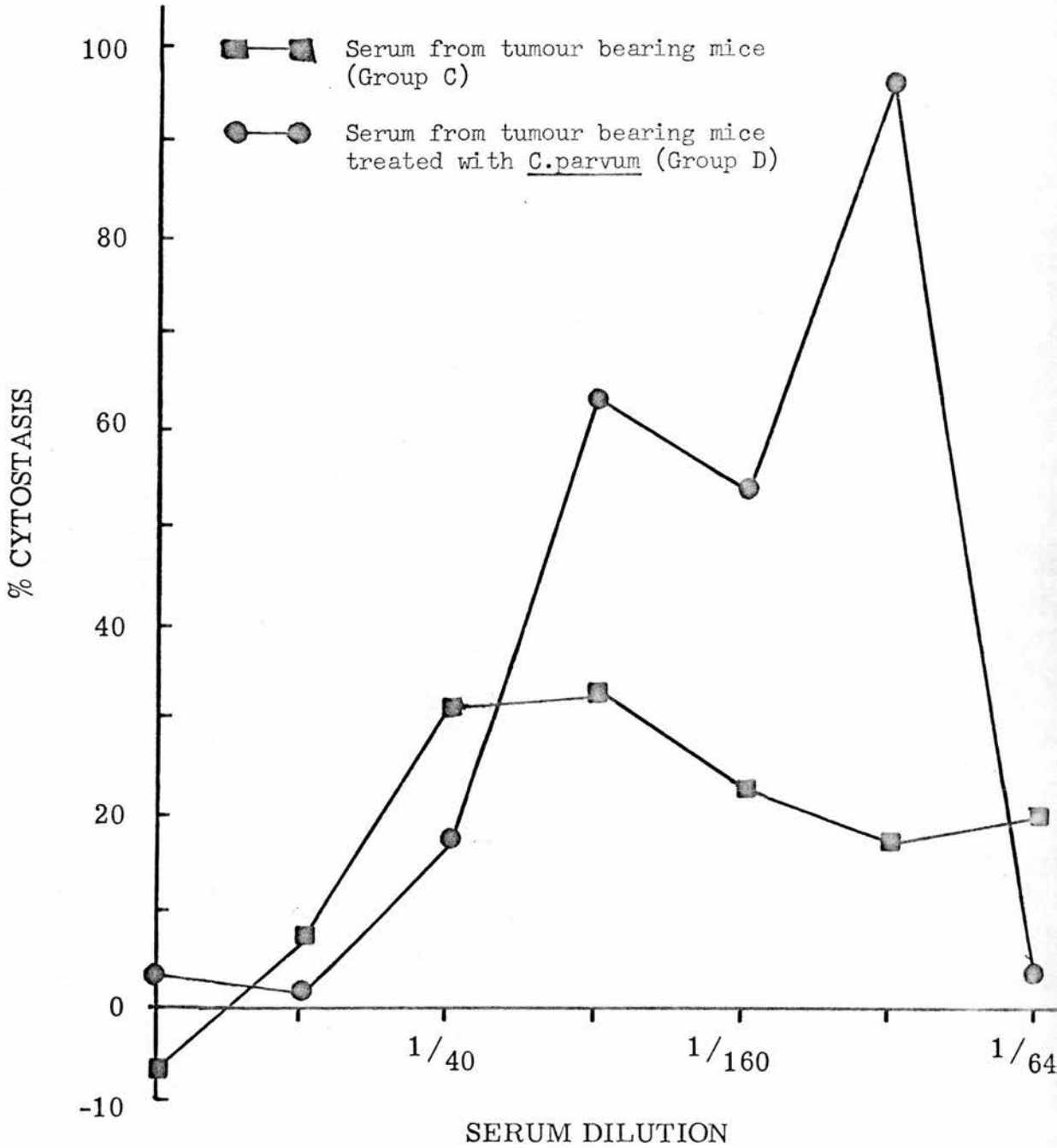
1) Group A - Normal CBA mouse serum
Group B - 1·4 mg C.parvum i.p. Day 0; mice bled Day 21

2) In this experiment serum from normal CBA mice was active in the cytostasis assay and was therefore not used as control serum in the equation to compute % cytostasis. A complement only control was used to compute the % cytostasis for serum from Groups A and B.

3) The % cytostasis obtained with serum from C.parvum-treated mice was higher and occurred over a greater dilution range than with serum from normal mice.

Fig. 38

CYTOSTATIC ACTIVITY AGAINST CBA FIBROSARCOMA CELLS OF SERUM FROM a) MICE BEARING A SYNGENEIC FIBROSARCOMA, b) MICE BEARING THE FIBROSARCOMA, TREATED WITH C.PARVUM



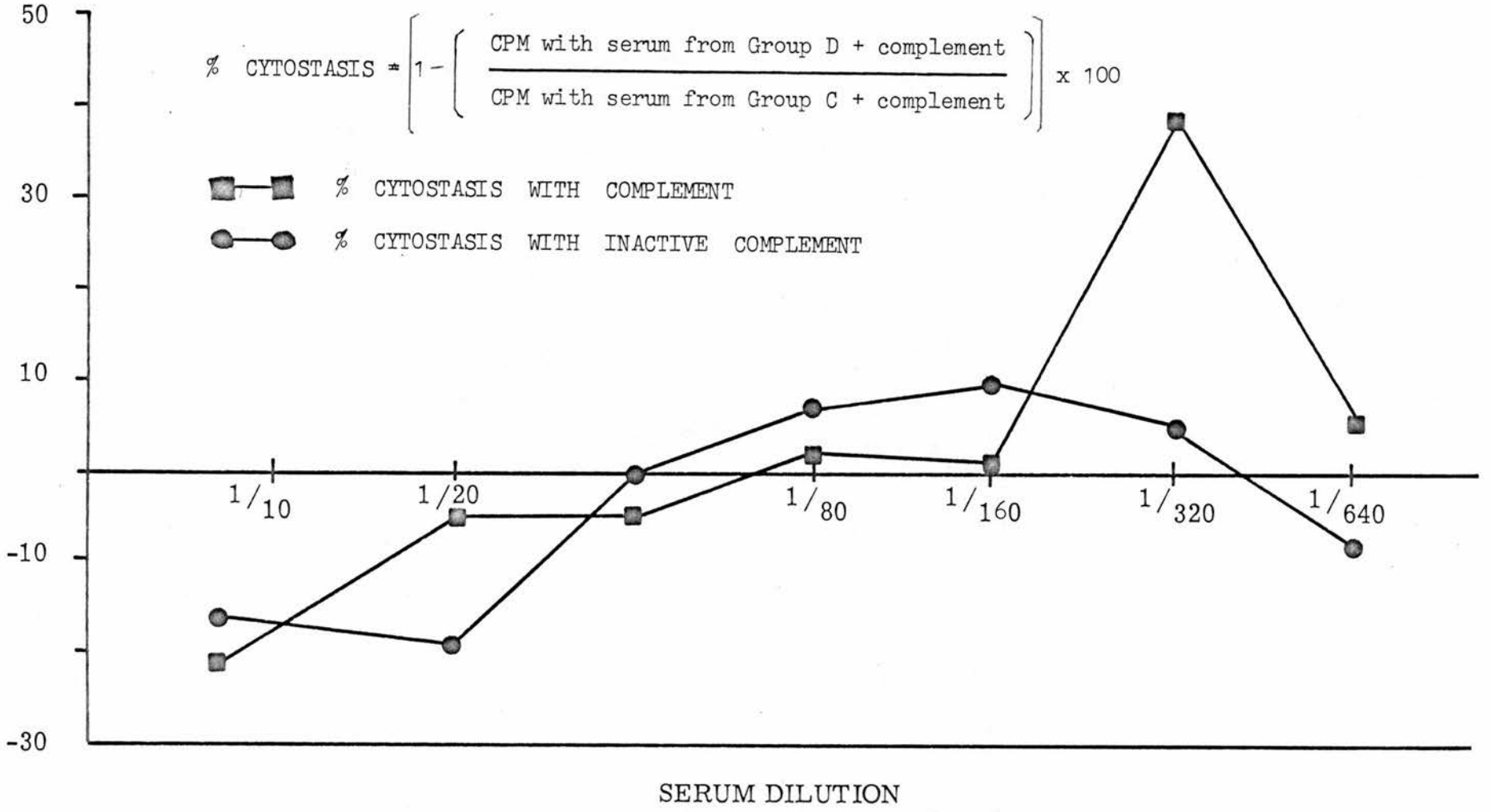
Note

1) Group C - 1×10^5 CBA fibrosarcoma cells s.c. Day 0
Group D - 1×10^5 CBA fibrosarcoma cells s.c. Day 0
1·4 mg C.parvum i.p. Day 3; mice bled Day 24

2) In this experiment serum from tumour bearing mice was active in the cytostatic assay and therefore was not used as control serum in the equation to compute % cytostasis. Instead a complement only control was used to compute the % cytostasis for Groups C and D.

3) The % cytostasis obtained with serum from C.parvum-treated tumour bearing mice was higher and occurred over a greater dilution range than with serum from tumour bearing mice.

Fig. 39 DEPENDENCE OF THE CYTOSTATIC EFFECT OF SERUM FROM C.PARVUM-TREATED TUMOUR BEARING CBA MICE ON EXTRANEOUS COMPLEMENT



Note (Fig. 39)

1) Group C - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0;
Group D - 1 x 10^5 CBA fibrosarcoma cells s.c. Day 0;
1·4 mg C.parvum i.p. Day 3; mice bled Day 24.

2) In this experiment serum from tumour bearing mice was inactive in the cytostasis assay against CBA fibrosarcoma cells and was therefore used as control serum in the equation to compute % cytostasis.

3) With active guinea pig complement only one serum dilution showed marked cytostatic activity. Inactivating the complement abolished this effect.

$$\% \text{ CYTOSTASIS} = \left[1 - \frac{\text{COUNTS PER UNIT TIME WITH SERUM FROM TUMOUR BEARING MICE TREATED WITH C.PARVUM + COMPLEMENT}}{\text{COUNTS PER UNIT TIME WITH SERUM FROM TUMOUR BEARING MICE + COMPLEMENT}} \right] \times 100$$

The results in Figs. 37 - 39 support the antiglobulin data in showing that there is an antigen-antibody reaction between CBA fibrosarcoma cells and serum from C.parvum-treated mice. However, it is not possible to say that the antibody detected by the antiglobulin assay is identical to the antibody detected in the cytostatic assay as the detailed specificity studies which characterised the former have not been carried out with the latter.

6. IMMUNOTHERAPY BASED ON THE PRECEEDING RESULTS

The goal of all cancer research is to establish suitable therapeutic regimes to control tumour growth. With this in mind we used the classical immunological modalities of active and passive immunisation a) to see if the recognition of embryonic antigens, indicated by Table 29, could be of use in immunising against a challenge of viable tumour cells; b) to see if antibody produced by C.parvum administration, and known to bind in vitro to CBA fibrosarcoma cells, could influence the growth of a CBA fibrosarcoma graft in syngeneic mice.

a) Active immunisation with embryo cells

It is a routine procedure in this laboratory to prevent the growth of an inoculum of CBA fibrosarcoma cells in syngeneic mice by a prior injection of X-irradiated fibrosarcoma cells. It was of interest to see if syngeneic embryonic cells could substitute for the immunising

dose of tumour cells in this system. Thus female CBA mice were immunised with either syngeneic X-irradiated or viable embryonic fibroblasts (1×10^6) s.c. on the right flank. Two weeks later the mice were challenged on the opposite flank with either freshly excised pronase-digested CBA fibrosarcoma cells or CBA fibrosarcoma cells grown *in vitro* for 2 - 3 weeks.

Unfortunately in two experiments the results were totally discordant. In the first experiment (Table 37) embryo cells appeared to immunise against a challenge of *in vitro* cultured cells but not against freshly excised pronase-digested cells. In the second experiment (Fig. 40) tumours grew in all groups and the rate of growth was only marginally slower in the groups immunised with embryo cells. Reasons for this discrepancy will be considered in the Discussion.

b) Passive immunisation with serum from *C.parvum*-treated mice

The passive transfer of serum took the form of transplanting tumour cells which had been incubated in medium alone or in serum from various experimental groups. Transplantation was via the i.v. or s.c. route and then either the number of tumour cell lung colonies was enumerated, or the tumour diameters measured, after a suitable time.

It can be seen that tumour cells treated with serum from *C.parvum*-treated mice could on occasions grow better than cells incubated with control serum. Incubation with such serum never produced diminished tumour growth (Table 38). The adverse effect was more apparent after i.v. injection and when the lungs were not overgrown with tumour.

TABLE 37

COMPARISON OF CBA FIBROSARCOMA CELLS AND SYNGENEIC EMBRYO CELLS AS IMMUNISING AGENTS AGAINST THE CBA FIBROSARCOMA

Immunisation With CBA Fibrosarcoma Cells

IMMUNISATION[a] (Day -14)	CHALLENGE[b] (Day 0)	TUMOUR TAKE (Day 33)
None	5×10^3 pronase digested tumour cells	$4/6$ (67%)
None	5×10^3 cultured tumour cells (47 days)	$3/7$ (43%)
$10^6 \times R$[c] tumour cells	5×10^3 pronase digested tumour cells	$0/5$ (0%)
$10^6 \times R$ tumuur cells	5×10^3 cultured tumour cells (47 days)	$0/6$ (0%)

Immunisation With CBA Embryonic Cells

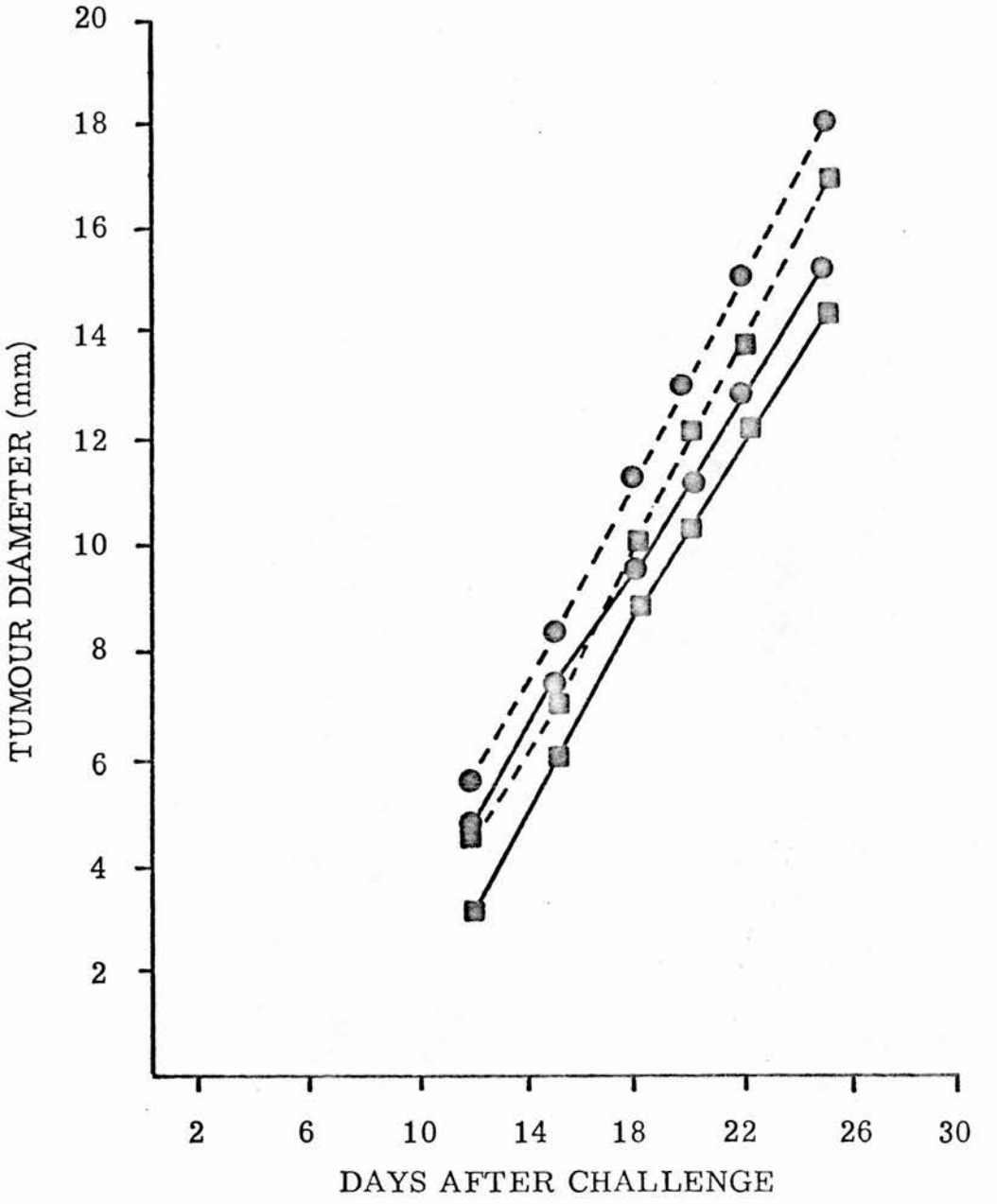
IMMUNISATION[a] (Day -13)	CHALLENGE[b] (Day 0)	TUMOUR TAKE (Day 35)
None	1×10^4 pronase digested tumour cells	$5/7$ (71%)
None	1×10^4 cultured tumour cells (21 days)	$4/7$ (57%)
1×10^6 viable embryonic cells	1×10^4 pronase digested tumour cells	$5/7$ (71%)
1×10^6 viable embryonic cells	1×10^4 cultured tumour cells (21 days)	$2/6$ (33%)
$1 \times 10^6 \times R$ embryonic cells	1×10^4 pronase digested tumour cells	$6/7$ (86%)
$1 \times 10^6 \times R$ embryonic cells	1×10^4 cultured tumour cells (21 days)	$0/7$ (0 %)

a) Cells for immunisation were prepared by enzyme digestion (see Materials and Methods).

b) Cells for challenge were either (1) freshly excised from a tumour bearing mouse, then pronase digested to obtain a single cell suspension; (2) obtained from an *in vitro* culture which produced a single cell suspension without enzyme digestion.

c) x R = X-irradiated.

Fig. 40 EFFECT OF IMMUNISATION WITH EMBRYO CELLS ON THE GROWTH OF A SYNGENEIC FIBROSARCOMA IN CBA MICE



Key

Immunisation	Challenge
CBA embryo cells	CBA fibrosarcoma (cultured in vitro)
None	"
CBA embryo cells	CBA fibrosarcoma (freshly excised pronase digested)
None	"

Note (Fig. 40)

1) CBA mice were immunised s.c. with 1 x 10^6 X-irradiated syngeneic embryonic cells and challenged on the opposite flank with 1 x 10^4 CBA fibrosarcoma cells (freshly excised pronase digested or *in vitro* cultured)

2) No inhibition of growth was observed for either freshly excised or *in vitro* cultured tumour cells in this experiment.

TABLE 38

THE EFFECT OF PRETREATMENT[a] WITH CBA MOUSE SERUM ON THE GROWTH OF TRANSPLANTED SYNGENEIC TUMOUR CELLS

EXP.	SERUM POOL	TUMOUR INJECTION: ROUTE	TUMOUR INJECTION: NOS. OF VIABLE CELLS	TUMOUR DIAMETER OR NOS. OF LUNG NODULES[b] AFTER PRETREATMENT WITH: MEDIUM	UNTREATED MOUSE SERUM (A)	C.PARVUM SERUM (B)	TUMOUR MOUSE SERUM (C)	TUMOUR C.PARVUM SERUM PLUS MOUSE (D)
i	1	SC	1×10^4	13·6 (12·0-15·4)	11·7 (9·6-14·2)	13·2 (11·6-14·9)	13·7 (11·7-15·9)	17·6 (16·6-18·8)
ii	1	SC	5×10^4	14·5 (12·7-16·5)	14·7 (14·4-15·1)	14·0 (13·6-14·4)	14·5 (14·1-14·8)	15·0 (14·6-15·5)
iii	2	SC	5×10^4	15·8 (14·9-16·8)	17·0 (16·7-17·3)	15·9 (14·6-17·3)	16·8 (16·1-17·6)	14·6 (13·6-15·7)
iv	1	IV	5×10^4	not determined	15 (11-20)	45 (37-53)**[c]	54 (43-67)**	81 (71-93)***
v	1	IV	5×10^4	33 (26-42)	43 (35-52)	86 (69-106)	34 (25-44)	98 (88-109)**
vi	1	IV	5×10^4	144 (108-192)	167 (145-192)	209 (195-295)	153 (130-182)	151 (132-173)
vii	2	IV	5×10^4	107 (96-119)	174 (152-197)*	129 (115-144)	104 (88-122)	134 (121-148)
viii	2	IV	5×10^4	269 (254-285)	305 (288-323)	284 (249-327)	238 (218-260)	222 (204-240)

a) Cultured syngeneic methylcholanthrene induced fibrosarcoma cells were incubated in dilutions (x 5) of serum for 45 mins. at 37°C and then injected s.c. or i.v. No extraneous complement was added during incubation.

b) Values for tumour diameters and nos. of nodules observed 22 to 29 and 14 days respectively following tumour transplantation.

c) Values in exp. iv compared with those observed in pool 1A; in all other experiments compared with medium only controls.

* denotes $P \leq 0.05$, ** denotes $P \leq 0.01$, *** denotes $P \leq 0.001$.

Note

1) CBA mouse serum from: Group A - untreated CBA mice; Group B - 1·4 mg C.parvum i.p. Day 3; Group C - 1×10^5 CBA fibrosarcoma cells s.c. Day 0; Group D - 1×10^5 CBA fibrosarcoma cells s.c. Day 0, 1·4 mg C.parvum i.p. Day 3. Mice bled Day 24.

2) On no occasion did preincubation in any of the sera significantly inhibit tumour growth. Indeed on occasions the sera from C.parvum-treated mice enhanced growth, see exp. iv and v. This was particularly apparent when the number of lung nodules was small.

7. ASPECTS OF THE IMMUNE RESPONSE IN MICE BEARING PLASMA CELL TUMOURS

One of the ways in which a neoplastic cell could grow progressively would be for the tumour to non-specifically depress the immune response of the host. Evidence from this laboratory indicated that the CBA fibrosarcoma might indeed inhibit the immune response in tumour bearing mice to defined antigens (183). However, the inhibition was confined to the response against alum-BSA (alum-precipitated bovine serum albumin); the response to SRBC and type III pneumococcus polysaccharide antigen being unchanged or elevated. On analysis of the sub-class of antibody involved in the anti-SRBC response, it appeared that the IgG_1 response was depressed if the antigen and tumour were injected simultaneously, whereas IgM and IgG_{2b} responses were elevated with an established tumour (184).

It was of interest to see if these results could be extended to tumours of lymphoid origin and if antibody-dependent lymphocyte cytotoxicity (K-cell activity) was affected by the presence of a lymphoid tumour transplant. These experiments were carried out in BALB/c mice using syngeneic plasmacytomas (see Table 1).

a) Effect of syngeneic transplanted plasmacytomas on the antibody response of BALB/c mice

We decided to investigate the antibody response to SRBC and alum-BSA. The effect of plasmacytoma growth on the anti-SRBC response was analysed in terms of splenic plaque-forming cells (PFC) of IgM, IgG_1, IgG_{2a} and IgG_{2b} classes and sub-classes, and the anti-alum-BSA response in terms of Antigen Binding Capacity (ABC) and Relative Binding Affinity (RBA).

Standard immunising doses of 1 mg BSA in alum, or 3 x 10^8 SRBC were used and all injections were by the i.p. route. Mice received alum-BSA 5 days after s.c. tumour transplantation (see Materials and Methods 3b). Immunisation with SRBC was either at the time of s.c. tumour transplantation or when the tumour was established. In this latter case immunisation was delayed until it was judged that the mice were within 14 days of succumbing to the lethal effects of tumour growth. Owing to variations in growth rate and number of cells transplanted (3 x 10^6 - 3 x 10^7) this was not at a standard time after tumour transplantation. The response to alum-BSA was measured 14 days, and the response to SRBC 7 days, after injection of the respective antigens.

The results in Table 39 show that the growth of all plasmacytomas significantly depressed the antibody response to alum-BSA as measured in terms of Antigen Binding Capacity. The splenic response to SRBC in mice simultaneously transplanted with tumour cells was depressed with 3 out of 4 plasmacytomas (Fig. 41). This was most apparent with the IgG_1 response. During the late growth of tumours none of the responses were significantly depressed and some were actually elevated. Again this was most apparent with the IgG_1 response.

b) Effect of syngeneic transplanted plasmacytomas on K-cell cytotoxic activity in the spleens of tumour bearing mice

The effect of an established plasmacytoma on the splenic K-cell activity against antibody-coated chicken red blood cells was examined as follows. Groups of BALB/c mice were injected s.c. with plasmacytoma cells prepared by homogenisation of solid tumour (see Materials

and Methods 3b). When the tumour was established and the mice looked to be within 14 days of succumbing they were killed by cervical dislocation and their spleens assayed for K-cell activity as described in Materials and Methods 4e. The number of viable tumour cells injected and the time of K-cell assay are shown in Tables 40 - 43.

Also measured in these experiments were the number of Fc-receptor bearing cells in each plasmacytoma (by the method of Szymaniec and James (102)), splenic hyperplasia, and tumour cell infiltrate to the spleen. The latter could be visualised when homogenised spleen cells from tumour bearing mice were centrifuged, the tumour cells sedimenting before the erythrocytes and forming a white layer upon which the red cells were supported. The height of this white layer gave a rough measure of the tumour cell infiltrate to the spleen. When these spleen homogenates were injected s.c. into syngeneic BALB/c mice they gave rise to tumours.

The results show that splenic K-cell activity was increased despite, or perhaps because of, a progressing plasmacytoma (Tables 40 - 43). Furthermore, some tumours appeared to metastasize to the spleen (ADJPC5 and MPC25). Interestingly, MOPC47A did not metastasize, and the tumour had a high proportion of Fc-receptor bearing cells, probably macrophages. However, MOPC104E had a low number of Fc-receptor bearing cells and also did not metastasize.

TABLE 39 INFLUENCE OF TRANSPLANTED PLASMACYTOMAS ON THE RESPONSE TO ALUM-BSA

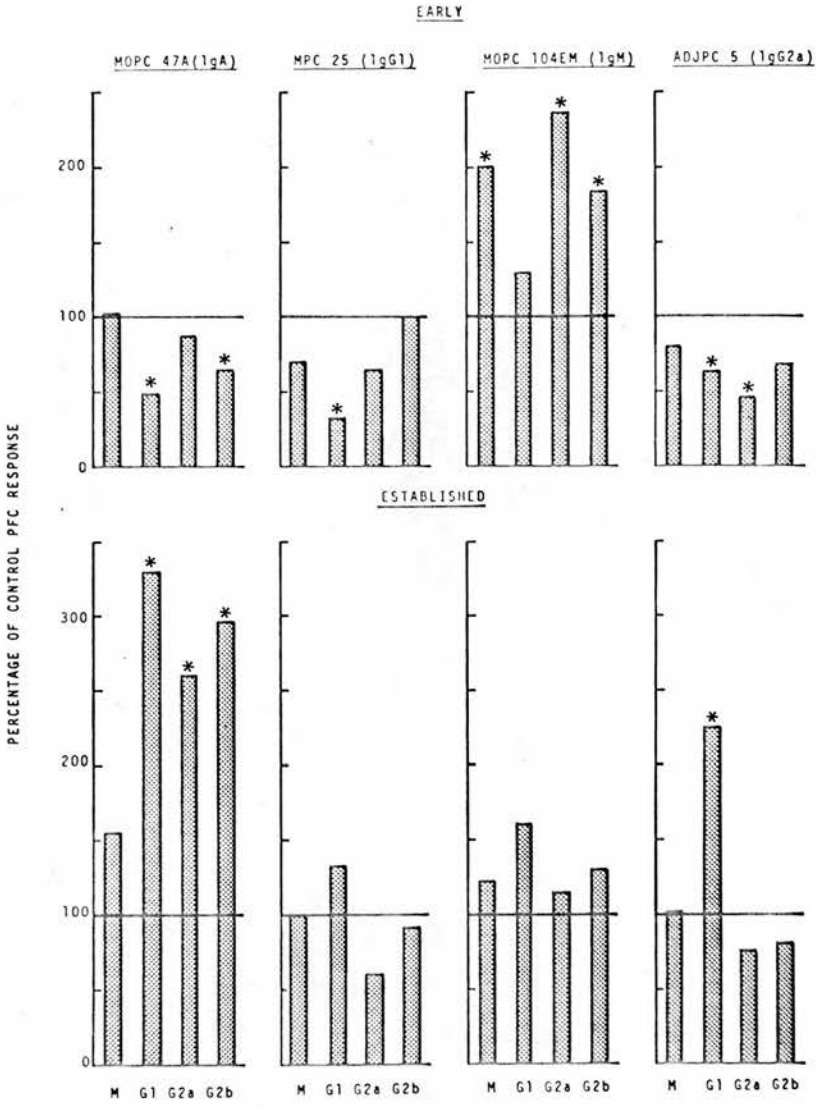
EXP.	PLASMACYTOMA[a]	ANTI-BSA RESPONSE		TUMOUR SIZE[b]		NUMBER OF ANIMALS
		ABC[c]	RBA[d]	DIAMETER (mm)	% BODY WEIGHT	
A	None	2·61 (2·17-3·14)	26·7 ± 1·9			10
A	MOPC 104EM (IgM)	1·27[e] (1·10-1·48)	23·5 ± 1·1	25·2 (23·5-26·9)	17·4 (15·5-19·7)	7
A	ADPC 5 (IgG_{2a})	0·67[e] (0·55-0·82)	27·9 ± 4·7	22·7 (21·2-24·3)	18·0 (15·0-21·6)	5
B	None	3·53 (3·23-3·86)	26·0 ± 3·2			6
B	MOPC 47A (IgA)	1·31[e] (1·09-1·58)	24·4 ± 3·4	15·5 (13·9-17·4)	6·85 (5·0-9·3)	6
B	MPC 25 (IgG_1)	1·01[e] (0·88-1·17)	39·7 ± 6·3	22·3 (21·6-23·1)	14·3 (12·8-15·9)	7

a) Inoculated s.c. 5 days before administration of alum-BSA.
b) At time of sacrifice.
c) Antigen Binding Capacity (0·2 µg BSA nitrogen per test) as geometric mean together with the limits of 1 S.E.
d) Relative Binding Affinity (arithmetic mean ± 1 S.E.)
e) Values significantly less than for controls.

Note

1) Values are for groups of BALB/c mice 14 days after 1 mg of alum-BSA i.p.
2) The growth of all plasmacytomas significantly depressed the antibody response to alum-BSA.

Fig. 41 INFLUENCE OF PLASMACYTOMA GROWTH ON THE RESPONSE OF MICE TO SRBC



Note

1) The splenic response (in terms of PFCs of IgM, IgG_1, IgG_{2a} and IgG_{2b} specificity) was measured 7 days after 3×10^8 SRBC were administered i.p. to BALB/c mice.

2) The response was expressed as a percentage of the control value (ie. mice without growing plasmacytomas).

3) SRBC were injected at the time of tumour inoculation (early) or at the time when tumour had grown (established).

4) * denotes a response significantly different to controls.

5) 3 out of 4 "early" plasmacytomas inhibited the PFC response - particularly the IgG_1 response. Established plasmacytomas either enhanced the PFC response or left it unchanged - again the IgG_1 response was the most enhanced.

TABLE 40 K-CELL ACTIVITY IN MICE BEARING MOPC47A PLASMACYTOMA[a]

		SPLEEN WT.(g)	TUMOUR WT. (% BODY WT.)	NUCLEATED CELLS PER SPLEEN (x 10^{-8})	TUMOUR CELL INFILTRATE TO SPLEEN	Fc-RECEPTOR Fc BEARING CELLS IN TUMOUR (%)	K-CELL ACTIVITY IN SPLEEN (%)			
							50/1[b]	20/1	10/1	5/1
TUMOUR BEARER	1	0·23	11	3·6	-	66	73	58	51	NT
	2	0·25	14	3·2	-	59	83	64	46	
	3	0·21	7	2·2	-	68	65	40	43	
	4	0·22	10	2·9	-	NT[c]	65	51	40	
CONTROL	1	0·12		1·6			68	40	48	NT
	2	0·13		1·5			48	32	48	
	3	0·12		1·4			50	38	35	
	4	0·13		1·4			41	35	41	
	5	0·12		1·5			40	38	49	

a) 3×10^5 viable plasmacytoma cells injected Day 0; parameters measured Day 20.

b) Effector to target cell ratio.

c) NT - not tested

Note

1) K-cell activity in spleens of tumour bearing mice greater than controls at effector/target cell ratios of 50/1 and 20/1.

2) High percentage of Fc-receptor bearing cells in this tumour.

TABLE 41 K-CELL ACTIVITY IN MICE BEARING MOPC10LE PLASMACYTOMA[a]

		SPLEEN WT.(g)	TUMOUR WT. (% BODY WT.)	NUCLEATED CELLS PER SPLEEN ($\times 10^{-8}$)	TUMOUR CELL INFILTRATE TO SPLEEN	Fc-RECEPTOR Fc BEARING CELLS IN TUMOUR (%)	K-CELL ACTIVITY IN SPLEEN (%)			
							50/1[b]	20/1	10/1	5/1
TUMOUR BEARER	1	0·20	9	2·5	-	5	49	HIGH BACKGROUND		
	2	0·27	13	4·3	-	2	73			
	3	0·29	13	3·3	-	4	73			
	4	0·25	8	3·4	-	NT[c]	70			
CONTROL	1	0·13		1·4			NT	HIGH BACKGROUND		
	2	0·11		1·1			20			
	3	0·11		1·2			20			
	4	0·13		1·2			23			
	5	0·15		1·4			42			

a) $1{\cdot}2 \times 10^6$ viable plasmacytoma cells injected Day 0; parameters measured Day 30.

b) Effector to target cell ratio.

c) Not tested.

Note

K-cell activity in spleens of tumour bearing mice greater than controls at effector/target cell ratio of 50/1

TABLE 42 K-CELL ACTIVITY IN MICE BEARING MPC25 PLASMACYTOMA[a]

		SPLEEN WT.(g)	TUMOUR WT. (% BODY WT.)	NUCLEATED CELLS PER SPLEEN (x 10^{-8})	TUMOUR CELL INFILTRATE TO SPLEEN	Fc-RECEPTOR Fc BEARING CELLS IN TUMOUR (%)	K-CELL ACTIVITY IN SPLEEN (%)			
							50/1[b]	20/1	10/1	5/1
TUMOUR BEARER	1	0·76	23	5·8	+	1	NT	38	25	22
	2	1·21	21	8·2	+++	4		61	45	33
	3	0·86	9	8·5	++	5		42	29	38
	4	0·30	4	3·6	+	NT[c]		29	32	32
CONTROL	1	0·13		1·4			NT	21	29	32
	2	0·13		1·3				27	40	20
	3	0·12		1·1				27	37	34
	4	0·13		1·5				20	18	25
	5	0·13		1·4				17	26	33

a) $1{\cdot}2 \times 10^6$ plasmacytoma cells injected Day 0; parameters measured Day 27.

b) Effector to target cell ratio.

c) NT - not tested.

Note

1) K-cell activity in spleens of tumour bearing mice greater than controls at effector/target cell ratio of 20/1.

2) Metastatic spread to spleen.

TABLE 43 K-CELL ACTIVITY IN MICE BEARING ADJPC5 PLASMACYTOMA[a]

		SPLEEN WT.(g)	TUMOUR WT. (% BODY WT.)	NUCLEATED CELLS PER SPLEEN ($\times 10^{-8}$)	TUMOUR CELL INFILTRATE TO SPLEEN	Fc-RECEPTOR Fc BEARING CELLS IN TUMOUR (%)	K-CELL ACTIVITY IN SPLEEN (%)			
							50/1[b]	20/1	10/1	5/1
TUMOUR BEARER	1	0·77	17	7·4	++	7	52	28	21	
	2	0·58	17	7·0	+	5	66	43	18	
	3	0·30	7	4·1	-	6	80	52	21	NT
	4	0·36	8	5·0	-	NT[c]	73	42	27	
	5	0·62	11	5·8	+	NT	60	36	20	
CONTROL	1	0·13		1·4			30	14	4	
	2	0·13		1·4			43	18	14	
	3	0·11		1·3			42	20	10	NT
	4	0·12		1·3			34	6	0	
	5	0·12		1·3			32	14	8	

a) $1{\cdot}2 \times 10^6$ plasmacytoma cells injected Day 0; parameters measured Day 21.

b) Effector to target cell ratio.

c) NT - not tested.

Note

1) K-cell activity in spleens of tumour bearing mice greater than controls at effector/target cell ratios of 50/1, 20/1, 10/1.

2) Metastatic spread to spleen.

DISCUSSION

1. CRITIQUE OF EXPERIMENTAL METHODS

It has often been said that in tumour immunology the technique adopted is everything. It is therefore appropriate that the advantages and disadvantages of the antiglobulin technique should be discussed here.

The results obtained with the antiglobulin assay (CPM, Absorption Ratio) vary with the cell number, antiglobulin specific activity, dilution of reagents, and possibly with the day to day condition of target cells. Therefore, a direct inference from CPM of the absolute amount of serum immunoglobulin bound to cells would be unreliable. The results in CPM of the test serum must be compared with a suitable control serum, in this case normal mouse serum from age matched controls.

However, as the serum of adjuvant treated mice contains greater amounts of immunoglobulin than untreated mice (see e.g. Fig. 34), it can be argued that untreated normal mouse serum is not an adequate control for non-specific adherence of immunoglobulin to cells. This brings up the issue of the specificity of the reaction, which can only be adequately settled by using a range of malignant and non-malignant target cells (see e.g. Tables 29 - 31). Normal rabbit serum or normal goat serum were used as controls for the specificity of the rabbit anti-mouse IgG (Table 28) and for the goat anti-mouse immunoglobulin antisera (Table 27), respectively. The specificity of the ^{125}I-globulin fraction of goat anti-rabbit IgG was apparent when compared

with ^{125}I-globulin fraction of normal goat serum (not shown). If the mouse serum was omitted (medium only control) the CPM was 20% of the CPM using normal serum (Fig. 28). However, if G-200 fractions were used the percentage could be greater.

In retrospect, how could the assay be improved? These studies have witnessed a considerable change in the antiglobulin assay from that originally used (see Table 10), and it finally gave results which were both coherent and consistent. However, this is not to say it could not be improved still further. For instance, although the rabbit anti-mouse IgG was absorbed with tumour cells, the antiglobulin reagent (goat anti-rabbit IgG) was not. This could account for part of the uptake of ^{125}I-labelled goat anti-rabbit IgG onto target cells; on the other hand it could be due to non-specific "stickiness".

Non-specific effects are a constant problem, and can lead to spurious conclusions if adequate controls are not incorporated into the experiment. It would, theoretically, be possible to overcome non-specific effects by producing a homogeneous antibody by means of immunoabsorbent columns, and using it in the antiglobulin assay. This has been tried using a single indicator reagent with some success (Figs. 16 and 33), although the logistic problems involved if the double layer technique were used would be formidable.

Such manipulations as the above might be expected to increase the sensitivity of the assay dramatically, However, more modest improvement in the specificity of uptake of mouse immunoglobulin <u>in vitro</u> might be achieved by altering the dilutions of mouse serum used. It is therefore realised that although the antiglobulin technique, as

presently used, gives consistent results, it may not have reached its full potential.

Improvement might also be looked for when performing absorptions of serum. It is probably true that any antibody activity would be absorbed out non-specifically if enough material was used for absorption. Therefore, it would be preferable to titrate the absorption of antibody activity using increasing numbers of cells and/or absorptions. In addition spleen cells are not a truly comparable control when absorbing serum with tumour cells, as tumour cells have a considerably larger surface area.

The results presented should be examined with the above reservations. However, there are positive advantages to the antiglobulin assay, such as economy of reagents, and the facility to process numerous experimental samples. The greatest advantage is that the assay is totally objective. This is of particular value in tumour immunology where one is constantly measuring barely significant differences. Thus, although the phenomena measured might be quantitatively small, there can be no doubt as to their existance. Their interpretation, however, is a legitimate object of contention.

Another advantage of the antiglobulin technique is that it is a primary assay. This means that it measures the antibody participating in the initial antigen-antibody reaction and is independent of secondary phenomena such as complement fixation. Thus, the antiglobulin technique is capable of detecting antibody of all classes and sub-classes.

2. SUMMARY OF RESULTS

The antiglobulin assay has been used to measure mouse immunoglobulin binding to syngeneic tumour cells *in vitro*. These studies showed that an i.p. injection of *C.parvum* into CBA mice elicited an increased level of immunoglobulin binding to tumour cells *in vitro* and that this was observed whether the mice had progressing tumours or not. The effect was dependent on the dose and route of *C.parvum* injection, occuring only after injection of high doses by a systemic route (Figs. 17 and 18).

Studies on the cellular basis for the production of immunoglobulin binding to tumour cells *in vitro*, after *C.parvum* injection, showed that gold salts could abrogate the effect (Fig. 20), thereby implicating macrophages as it is known that gold salts inhibit phagocytic activity (181). Results in T-cell-deprived mice were inconclusive due to large variations within experimental groups; also no direct comparison with intact mice was made. However, comparing results from previous experiments with intact mice, the production of immunoglobulin binding to tumour cells *in vitro* appeared to be diminished in tumour bearing 'B'-mice (Fig. 21). Furthermore, the effect was still apparent when *C.parvum* was injected into "nude" mice (Fig. 22 and Table 23); in this case the "nude" trait was on a BALB/c background and the target cells were CBA fibrosarcoma.

Other work showed that various strains of *C.parvum*, and other ostensibly unrelated adjuvants, elicited immunoglobulin binding to tumour cells *in vitro*. Using BCG and *B.pertussis* (Fig. 27) the effect was of a lesser magnitude than for *C.parvum* CN 613h in normal

and tumour bearing mice. When using adjuvants related to C.parvum CN 6134 (Fig. 25) it was again found that they all give similarly low levels of immunoglobulin binding to tumour cells in vitro when administered to normal mice, but C.parvum 10387 give particularly high levels when injected into tumour bearing mice. Evidence is also presented that the binding of mouse immunoglobulin to tumour cells in vitro also occured in vivo (Figs. 23 and 24).

The class and sub-class of immunoglobulin binding to tumour cells in vitro were examined, and it was immediately obvious that there was a difference between the response of mice treated with C.parvum and mice treated with C.parvum plus CBA fibrosarcoma cells (Tables 26 and 27). This difference had not been evident when whole mouse serum and an antiserum reacting with all the mouse immunoglobulin classes and subclasses was used. Thus, it can be seen that a 7S as well as a 19S response is evoked in mice treated with C.parvum plus CBA fibrossrcoma cells, whereas only a 19S response is seen in animals treated with C.parvum only. The 19S response correlated with high anti-C.parvum antibody titres, but the 7S did not (Table 25).

Table 25 shows that mice bearing a syngeneic fibrosarcoma, and treated with C.parvum, mount a complex antibody response to syngeneic tumour cells. It was therefore of interest to see if the same specificity towards normal target cells was exhibited by the 19S and 7S responses. The raw data are presented in Tables 29 - 31 and are summarised in Table 32. This indicates a certain degree of specific antibody activity in serum from C.parvum-treated tumour bearing mice, for homologous tumour cells.

Specificity was also examined by absorption of serum with syngeneic fibrosarcoma cells, spleen cells and C.parvum. The results (Table 33) indicate that absorption of serum from C.parvum-treated CBA fibrosarcoma bearing mice, with CBA fibrosarcoma cells, effectively abolished the uptake of immunoglobulin in vitro, relative to serum from tumour bearing mice. Absorption with spleen cells was not so effective, perhaps due to the spleen cells not possessing the tumour-specific antigen, although other explanations are possible.

The above is a synopsis of the results obtained in Results 2, and on the grounds of consistency and reproducibility cannot be doubted. Results illustrated in Results 3, 5, 6 and 7 are more in the nature of preliminary experiments; in some cases an experiment may only have been carried out once, and in others the phenomenon may not have proved to be reproducible.

Following Thomson et al. (154) and Le Mevel et al. (155), an attempt was made to elicit anti-tumour antibody in the serum of mice by either amputating their tumours, or hyperimmunising them with syngeneic tumour cells. The antibody detected by Thomson et al. appeared to be specific when tested against a rather restricted range of target cells. On the contrary, Burdick et al. (45) showed, in an exemplary series of experiments, that the antibody in the latter study was directed against an embryonic antigen, expressed in varying amounts on different transformed cell lines.

The immunoglobulin binding to tumour cells in vitro is referred to as anti-tumour antibody in Results 3. This is because it might be expected a priori to be directed against tumour-associated antigens as

the mice had only been treated with syngeneic tumour cells.

As regards the production of anti-tumour antibody after amputation of a palpable tumour, the results are inconclusive. In the experiment which should have yielded the most significant results (Fig. 31), anti-tumour antibody was not produced after amputation of tumour when compared to sham-operated controls, although it was when compared to tumour bearing controls. The literature is also confusing on this point: thus Thomson et al. (154) detected a long lasting anti-tumour antibody after tumour amputation, whereas Burdick et al. (185) could not find the expected inverse correlation between possession of a progressing tumour and production of anti-tumour antibody.

The results concerning the production of anti-tumour antibody after hyperimmunisation with syngeneic tumour cells passaged in vivo are more consistent. Briefly, the production of anti-tumour antibody in this system needed a large number of tumour cells (Table 34), and it appeared to be specific for the tumour antigens of the fibrosarcoma cells used for immunisation (Fig. 32). In this system tumour bearer mouse serum did not contain tumour-specific antibody.

The results in section 4 are concerned with the changes in total immunoglobulin class and subclass levels in the serum of mice treated with either C.parvum, tumour or both. It can be seen that an i.p. injection of C.parvum, in otherwise untreated mice, resulted consistently in elevated levels of IgG_{2b}, generally accompanied by elevated IgG_{2a} and/or IgM levels (Fig. 34, Table 36). Mice with a progressing syngeneic fibrosarcoma invariably had lower levels of immunoglobulin

than normal mice; however, an i.p. injection of C.parvum in tumour bearing mice raised the immunoglobulin levels to approximately those in normal mouse serum, or higher.

These serological changes were route and dose dependent; thus, no elevation of immunoglobulin class and sub-class levels was observed after either s.c. injection of C.parvum or of small doses. In tumour bearing mice the elevated immunoglobulin levels, apparent after C.parvum administration, could be at least partially diminished by treatment with gold salts (Fig. 36).

Such changes, however, were thymus-independent as all immunoglobulin class and sub-class levels (except IgG_1) were raised after C.parvum administration to tumour bearing 'B'-mice (Table 22). In "nude" mice also (which had low levels of all immunoglobulins except IgM), C.parvum elicited a rise in immunoglobulin levels (Table 23). It is of interest that the level of serum IgG_1, the most thymus-dependent sub-class (141), is the least responsive to change after C.parvum administration.

The effects of C.parvum strains CN 6134 and 10387, and P.freudenreichii were examined for their effects on serum immunoglobulin levels. All of the adjuvants induced a significant increase in serum IgG_{2b} levels in normal and tumour bearing mice. Significant increases in IgM levels were also noted in all mice treated with C.parvum 10387 and P.freudenreichii (Table 35). In another experiment the abilities of BCG and B.pertussis to elicit increased serum immunoglobulin levels were compared to that of C.parvum CN 6134. Treatment with C.parvum and B.pertussis resulted in a marked increase in IgG_{2a} and

IgG_{2b} levels in normal mice. In addition they also caused a significant increase in the levels of all immunoglobulins (except IgA) in tumour bearing mice. The BCG protocol elicited a raised level of IgG_{2a} in normal mice, and of IgG_1 and IgG_{2b} in tumour bearing mice (Table 36).

The results in section 5 indicate that the serum of C.parvum-treated mice has antibody which is capable of binding to an antigen possessed by tumour cells, and thereby activating complement (Figs. 37 - 39). Furthermore, it is possible that normal mouse serum and serum from tumour bearing mice contain an antibody which reacts with tumour cells (Figs. 37 and 38).

These results are meant to be treated as preliminary for the following reasons: no information other than that shown is available; and the cytostatic effect was absent if the uptake of ^{125}I-iododeoxyuridine was low. Recent unpublished observations incline us to the view that a high uptake by target cells depends on certain serum factors being present in the ambient medium.

The results in section 6 show the outcome of attempts at therapy based on the results in section 2. It appeared from Table 37 that either a prior injection of X-irradiated embryo cells or X-irradiated tumour cells could confer protection on the syngeneic host against homologous tumour cells. It was well known that the CBA fibrosarcoma in question was immunogenic; however, as the possibility of immunisation with embryo cells was new, this part of the experiment was repeated. Unfortunately this second experiment yielded no evidence of protection by prior administration of X-irradiated embryo cells (Fig. 40).

Aside from the trivial explanation of experimental error, the following considerations might explain the discordant results: a) the occurrence of embryonic antigen is a phase-specific event, occurring maximally in the mid-gestation period (47); b) embryonic tissue derived from multiparous animals may be less likely to evoke tumour resistance compared with embryonic tissue from primiparous animals (186). It is of interest that, in another tumour model, the rejection responses in rats immunised with X-irradiated embryonic tissue were also reported to be inconsistent (186).

The transplantation of tumour cells preincubated with serum from C.parvum-treated mice also gave rather variable results. However, it was quite obvious that s.c. or i.v. implantation of pretreated tumour cells never resulted in inhibition of tumour growth, and under certain circumstances lead to enhanced growth (Table 38).

Section 7 recounts observations on the immune status of plasmacytoma bearing mice. The responses to alum-precipitated bovine serum albumin (alum-BSA) and sheep erythrocytes were assessed in BALB/c mice; in addition splenic K-cell activity was examined.

The growth of all the plasmacytomas tested significantly depressed the response of mice to alum-BSA in terms of the Antigen Binding Capacity, but had no effect on the Relative Binding Affinity of the antibody produced (Table 39).

The influence of early or established tumour on the response to SRBC, in terms of splenic plaque-forming cells of IgM, IgG_1, IgG_{2a} and IgG_{2b} specificities was variable. Early growth of MOPC47A (IgA), MPC25 (IgG_1) and ADJPC5 (IgG_{2a}) plasmacytomas depressed certain

IgG responses, especially IgG_1, while IgM responses were unaffected. In contrast the early growth of an IgM-secreting plasmacytoma (MOPC 104E) enhanced both IgM and IgG responses. Established plasmacytomas had no effect on the IgM responses to SRBC, although MOPC 47A and ADJPC5 gave some enhancement of certain IgG responses (Fig. 41).

In the study of splenic K-cell activity in plasmacytoma bearing mice the parameters measured again showed some variation between the different tumours. Thus, although an established plasmacytoma was always associated with enhanced splenic K-cell activity and splenomegally, the IgG secreting tumours ADJPC5 (IgG_{2a}) and MPC25 (IgG_1) exhibited a capacity to metastasize to the spleen. Furthermore, the IgA secreting plasmacytoma (MOPC 47A) exhibited a large percentage of Fc-receptor bearing cells (Tables 40 - 43).

3. RELEVANCE OF RESULTS TO TUMOUR IMMUNITY

The introduction dilated, at some length, on the genetic mechanisms underlying the immune response, and their augmentation by various means. The mice used in this tumour model appear to be genetically endowed with the capacity to respond to the tumour antigens in question, as evidenced by prior immunisation with X-irradiated cells. However, the response in unimmunised mice is ineffectual as they invariably succumb to the lethal effects of a growing tumour. C.parvum redresses the balance somewhat, but is this due to an increased immune response to tumour antigens?

Two mechanisms which could, hypothetically, mediate the anti-tumour effect of C.parvum have been discussed (see Introduction 6a).

These are a non-specific mechanism produced by systemic injection of adjuvant and a specific mechanism produced by contact between adjuvant and tumour cells. The latter has a respectable theoretical background in that it appears to be an augmentation of the host response to an antigen by adjuvant (120). On the other hand the non-specific mechanism requires the new hypothesis that macrophages have the capacity to discriminate between malignant and non-malignant cells (187).

There is doubtless something appealing about this hypothesis; indeed, it has been known since the days of Metchnikoff that macrophages could ingest foreign matter, at least matter that was grossly different from self. However, in a study on the effects of macrophages on a wide range of malignant and non-malignant cell lines, Keller reported that there was no correlation between susceptibility to cytostasis and degree of malignant transformation (188).

There was, however, a correlation between susceptibility to the cytostatic effect of macrophages and the rapidity of replication of target cells (187, 188). But, as rapid proliferation is not an exclusive attribute of malignant cells, there is an intuitive difficulty in seeing how non-specifically activated macrophages could exert a selective anti-tumour effect. This is not to say that in an artificial situation, say an injection of 1×10^5 tumour cells s.c., that the tumour cells would not be ingested by activated macrophages due to the anatomically anomalous position of the cell inoculum. In fact this appears to be the case, and the sequel is that a specific response to tumour antigens is mounted (120).

What is the bearing of the results presented here on the situation as outlined above? The salient point is that an injection of C.parvum can induce elevated levels of immunoglobulin binding to tumour cells in vitro. The production of this immunoglobulin appeared to be connected with the anti-tumour effect of C.parvum injected i.p., as high levels of immunoglobulin binding to tumour cells in vitro could be associated with a pronounced inhibition of tumour growth. Thus, an i.p. injection of C.parvum into tumour bearing mice produced inhibition of tumour growth and elevated levels of immunoglobulin binding to tumour cells in vitro; s.c. injection did neither. Similarly, the inhibition of tumour growth by C.parvum could be abrogated by gold salts, as could the production of immunoglobulin binding to tumour cells in vitro.

Later results indicated that although the production of immunoglobulin binding to tumour cells in vitro might be a necessary condition for inhibition of tumour growth, it was not sufficient. Thus, strains of C.parvum which elicited elevated levels of immunoglobulin binding to tumour cells in vitro did not inhibit tumour growth.

Both the production of immunoglobulin binding to tumour cells in vitro, and growth inhibition, after C.parvum injection appear to have some elements of their respective mechanisms in common, as they are both influenced by gold salts (ie. sodium aurothiomalate). The available evidence suggests that this anti-inflammatory agent is rapidly concentrated within phagocytic cells where it inhibits lysosomal enzyme activity (181). Furthermore, gold salts inhibited the appearance of cells which were non-specifically cytostatic for tumour cells

in vitro (Cullen and Ghaffar, unpublished). Thus, it appears extremely likely that gold salts depress macrophage function, leading to a corresponding change in the serological parameters measured.

The results in T-cell-deprived mice were inconclusive; nevertheless, the impression was gained that both inhibition of tumour growth, and production of immunoglobulin binding to tumour cells *in vitro*, were diminished compared to intact controls. This could have been due to the loss of the specific T-cell component in both cases.

A wide range of classes and sub-classes of immunoglobulin, from the serum of *C.parvum*-treated tumour bearing mice, are capable of binding to syngeneic tumour cells *in vitro*. This does not support the theory that only "blocking" antibody of a particular class or sub-class is produced in response to tumour antigens. However, it may be that adjuvant shifts the production of antibody from one class or sub-class to another. Certainly the immunoglobulin in the serum of mice treated with *C.parvum* plus tumour, binding *in vitro* to homologous tumour cells, is different to that in the serum of tumour bearing mice, both quantitatively and qualitatively. However, it is not known whether this is due to antigen-antibody complex formation in untreated mice with a tumour burden, or to an increased or *de novo* synthesis of immunoglobulin binding to tumour cells *in vitro*, after adjuvant administration to tumour bearing mice.

There are several conceivable explanations of the presence in the serum of *C.parvum*-treated mice of immunoglobulin binding to tumour cells *in vitro*. These include: a) non-specific "sticking" of immunoglobulin to cells; b) the production of increased levels of

cytophilic immunoglobulin which binds to Fc-receptors (163); c) the production of antibody against antigenic determinants shared by C.parvum and target cells (158); d) an increased production or de novo synthesis of autoantibody (189); e) an increase in the level of pre-existing anti-tumour antibody (157); f) the production of antibody against unique or common tumour antigens (44). Needless to say, such mechanisms are not mutually exclusive.

Non-specific sticking of immunoglobulin to cells is difficult to control (Discussion 1); therefore, it is not possible to say unequivocally that immunoglobulin from the serum of mice treated only with C.parvum binds to tumour cells in vitro via an antigen-antibody reaction. Certainly neither whole serum from C.parvum-treated mice (compared with normal serum), or their 19S fractions, exhibited any target cell specificity. However, the data from Results 5, which shows that the serum of C.parvum-treated mice can exert a cytostatic effect on CBA fibrosarcoma cells, indicates that the antiglobulin assay is measuring an antigen-antibody reaction.

As regards uptake of mouse immunoglobulin via Fc-receptors on target cells, there was every reason to suppose that there would be no Fc-receptor bearing cells in a cell population cultured in vitro for over two weeks, because the proportion of Fc-receptor bearing cells in a CBA fibrosarcoma cell suspension falls rapidly over two weeks from around 30% to 0% (102). Nevertheless, experiments were performed which confirmed that only when using target cells containing macrophages (ie. freshly prepared spleen cells) did blocking of uptake by aggregated human IgG occur.

If the trivial explanations a and b are discounted, there remains the mechanisms c, d, e and f, for the uptake of immunoglobulins onto tumour cells *in vitro*. The possibility of shared antigens between micro-organisms and certain tumour cells has been suggested for BCG (190) and *L.monocytogenes* (158). Cross-reactions between bacteria and normal tissue antigens have been reported (160), which may explain some of the results recorded here. Furthermore, a theoretical basis has been put forward (159). However, the question of whether micro-organisms possess antigens which are in any way specific for the neoplastic state of mammalian cells is debatable, and receives no support from the work presented here.

Before the idea is completely dismissed, it might be as well to ponder on the variability of tumour antigens, which arise in a seemingly random manner. It may be that chance would decree a cross-reaction between tumour cells and totally unrelated micro-organisms. Such a mechanism would be of severely limited benefit in immunotherapy, but may have a modest role in explaining certain empirical observations.

As well as acting as an immunological adjuvant *C.parvum* also elicits antibody to mouse red blood cells (189) and rheumatoid factor in some cancer patients on *C.parvum* therapy (136). Such factors may confuse the results of the antiglobulin assay. However, when it is considered that absorption with *C.parvum* effectively removes immunoglobulin binding to mouse target cells *in vitro*, there is really no evidence for the production of classical autoantibody by abrogation of tolerance after adjuvant administration, in these studies.

Antibody cytotoxic for various types of tumour cells has been

reported. Cytotoxic sera from "nude" mice were active against a range of tumour target cells, indicating the antibody was either directed against a common antigen (157) or that it contained a heterogeneous collection of specificities. The cytotoxic activity was not uniformly distributed in that some mouse strains were high responders and some low. Furthermore, an inverse relationship was demonstrated, in a given mouse strain, between cytotoxicity of serum and susceptibility of lymphoma cells to the cytotoxic effect (191). Thus, in the above studies serum was only cytotoxic towards allogeneic target cells, and was ineffective in syngeneic combinations.

This activity may or may not be directed against tumour associated antigens; however, a similar mechanism may explain the results showing the binding of immunoglobulin from normal mouse serum to tumour cells in vitro (Fig. 33). Support for this suggestion comes from the observation that normal mouse serum can be cytostatic for syngeneic tumour cells (Fig. 37).

The most interesting of the possibilities is the promotion by C.parvum of a response to tumour-specific antigens in tumour-bearing mice. As the most convincing evidence of a tumour-specific response is the use of other target cells as negative controls, we have examined the in vitro uptake of immunoglobulin from C.parvum-treated tumour bearing mice onto malignant and non-malignant target cells. To obtain a measure of the uptake due to a response to tumour-specific antigens it might be expected that a comparison of the in vitro uptake of immunoglobulin from the serum of mice treated with C.parvum and tumour, with that from the serum of mice treated with C.parvum only,

might be instructive. If this is done it can be seen that the 19S response exhibits no specificity for CBA fibrosarcoma cells. However, the 7S uptake is consistently greater for CBA fibrosarcoma cells than for any other target cells tested (Tables 29 - 32).

Other evidence for a tumour-specific antibody response in tumour bearing mice treated with C.parvum is: 1) the anti-C.parvum antibody titres in the 7S fractions of serum from C.parvum-treated mice and mice treated with C.parvum plus tumour are similar, but the immunoglobulin binding to tumour cells in vitro is much greater in the latter (Table 25); 2) IgM, IgG_1 and IgG_{2b} from the serum of mice treated with C.parvum plus tumour bind to tumour cells in vitro to a greater extent than similar immunoglobulins from the serum of mice treated with C.parvum only (Tables 26 and 27); 3) absorption with spleen cells leaves a "residue" of immunoglobulin still capable of binding to tumour cells in vitro (Table 33).

It may be that the tumour-specific antibody response is obscured by mechanisms a - e, which have been discussed previously. This may be similar to the system described recently (192) in which the killing of tumour target cells, by peritoneal cells from mice injected with BCG plus X-irradiated homologous tumour cells, was examined. In this system the peritoneal cells were non-specifically cytostatic for a range of target cells. However, when the glass-adherent cells were removed the remaining lymphoid cells were specifically cytotoxic for the tumour cells used for immunisation.

The results in sections 3 and 5 are to be seen as adjuncts to

those in section 2. Thus, section 3 indicates that CBA mice can respond to the syngeneic tumour by mounting a humoral response, albeit a comparatively small and at present ill-defined one. Section 5 indicates that the immunoglobulin binding to tumour cells in vitro, detected by the antiglobulin technique, may infact be due to an antigen-antibody reaction.

The work recounted in section 4 was undertaken in order to relate serum immunoglobulin class and sub-class levels to the tumour status of individuals with and without the benefit of systemic C.parvum therapy. In general the possession of a growing tumour lead to dramatic decreases of all immunoglobulin levels, particularly IgG_{2a} and IgG_{2b}, whereas administration of C.parvum to tumour bearing mice elevated them to the levels prevailing in untreated mice, or higher. The injection of C.parvum into normal mice also resulted in elevated immunoglobulin levels, and again this was most dramatic with IgG_{2a} and IgG_{2b}.

The mechanism of production of immunoglobulin after adjuvant administration has been investigated by Humphrey (1964) and Tam et al. (1966) for BCG. For this adjuvant it appears that the "response" is due to production of non-specific immunoglobulin, without the mark of a particular antigen. In addition, a delayed-type hypersensitivity reaction to constituents of the BCG is necessary, presumably implicating T-cells.

Our results are in line with the notion that there is a non-specific production of immunoglobulin after C.parvum administration, as exhaustive absorption of such serum with C.parvum does not reduce the serum

immunoglobulin levels to control values (not shown). However, a fully functional thymus is not necessary as the elevated serum immunoglobulin levels are seen in T-cell-deprived mice treated with C.parvum (Table 22). This is in accord with the idea that adjuvant action of C.parvum is more dependent on B-cells and/or macrophages than is BCG. (Note however that the antibody response to C.parvum is partially T-cell dependent (Table 23)).

In a recent study the effect of C.parvum therapy on serum immunoglobulin class and sub-class levels in cancer patients was investigated (136). Again the adjuvant showed some selectivity in its ability to elevate certain immunoglobulin sub-class levels, in this case IgG_2. This, however, may not be directly analogous to the results presented in section 4 as human IgG_2 is putatively the homologue of mouse IgG_1, which in general is least responsive (in terms of total serum concentration) to C.parvum.

It may be that the one high dose of C.parvum given to mice elicits a predominantly T-cell-independent effect, whereas the numerous low doses given to humans elicit a predominantly T-cell-dependent effect.

The data on serum immunoglobulin levels in individuals bearing tumours is contradictory. Our own studies show a decrease in immunoglobulin levels, from day 7 to day 21 after injection of tumour cells. On the contrary, Witz has reported that mice injected with a transplantable sarcoma have elevated levels of IgG_2 from 9 - 24 days after tumour transplantation (193).

The situation with regard to the immunoglobulin levels in cancer patients with a tumour burden is also confusing. Thus, it has been

TABLE 44 — THE EFFECTS OF C.PARVUM IN NORMAL CBA MICE : A SUMMARY

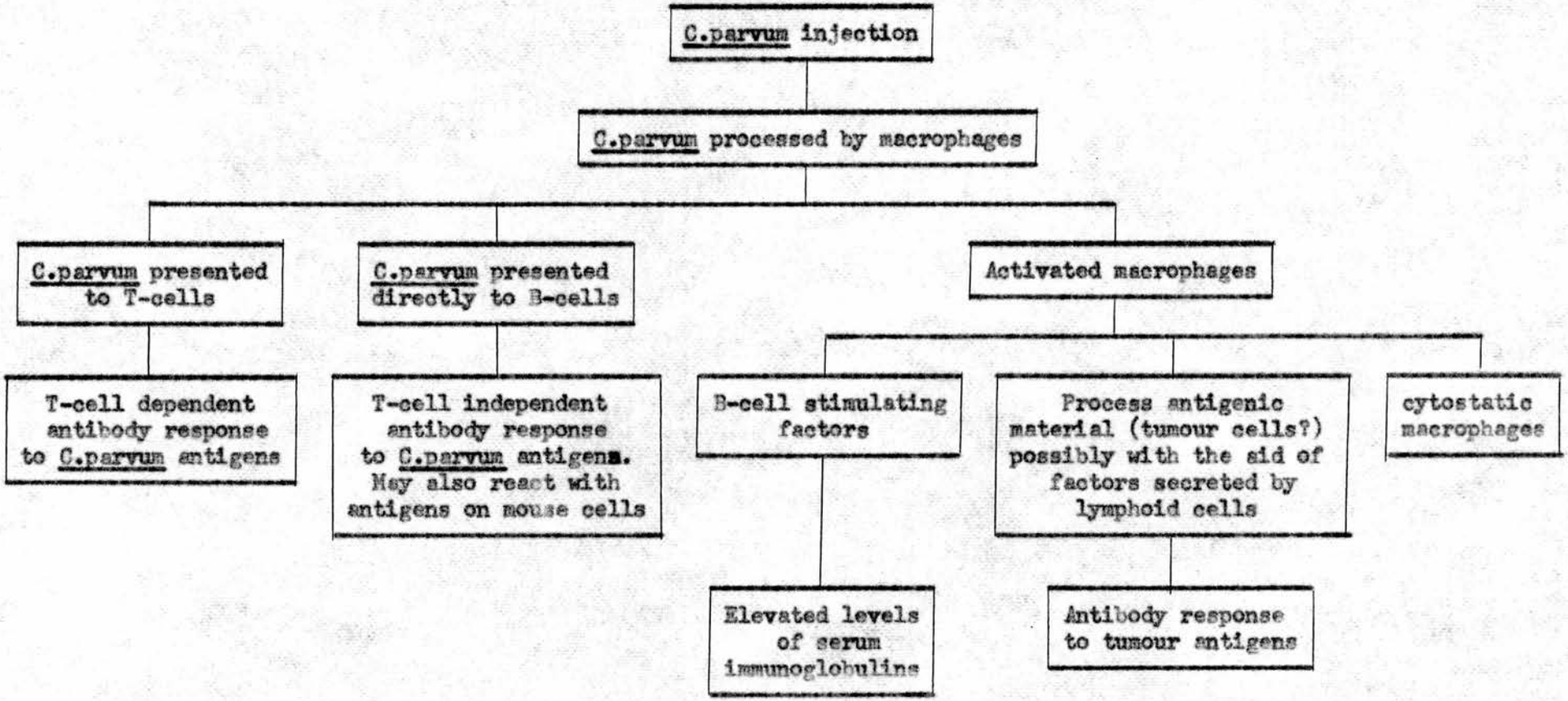
TREATMENT	PARAMETER MEASURED		
	Serum immunoglobulin class and sub-class	Immunoglobulin binding to tumour cells in vitro	Anti-C.parvum antibody
Injection of C.parvum by different routes (i.p. or s.c.)	i.p: IgG_{2a} ↑, IgG_{2b} ↑. s.c: →	i.p: ↑ s.c: →	i.p: ↑↑ s.c: ↑
Injection of different doses of C.parvum (i.p.)	1·4 mg : IgM ↑, IgG_{2b} ↑. Lower doses: →	0·7 mg & 1·4 mg: ↑ Lower doses: →	0·1hmg, 0·7mg, 1·4mg: ↑↑
Injection of different strains of C.parvum	CN 6134 : IgG_{2b} ↑. 10470 : IgG_{2b} ↑, IgM ↑. 10387 : IgG_{2b} ↑, IgM ↑.	CN 6134 : ↑ 10470 : ↑ 10387 : ↑	N.T. N.T. N.T.
Injection of different adjuvants	C.parvum : IgG_{2a} ↑, IgG_{2b} ↑. B.C.G. : IgG_{2a} ↑. B.Pertussis : IgG_{2a} ↑, IgG_{2b} ↑.	C.parvum : ↑ B.C.G. : → B.Pertussis : →	N.T. N.T. N.T.
Injection of C.parvum into "nude" mice (i.v. or i.p.)	i.v: IgM ↑, IgG_{2a} ↑ i.p: →	i.v: ↑ i.p: ↑	i.v: ↑ i.p: ↑

TABLE 45 THE EFFECTS OF C.PARVUM IN TUMOUR BEARING CBA MICE : A SUMMARY

TREATMENT	PARAMETER MEASURED			
	Serum immunoglobulin class and sub-class	Immunoglobulin binding to tumour cells in vitro	Anti-C.parvum antibody	Inhibition of tumour growth
Injection of C.parvum by different routes (i.p. or s.c.)	i.p: IgG_{2a} ↑, IgG_{2b} ↑, IgG_1 ↑, IgA ↑. s.c: all classes and sub-classes →.	i.p: ↑ s.c: →	i.p: ↑↑ s.c: ↑	i.p: ↑ s.c: →
Injection of C.parvum together with gold salts	C.parvum: IgG_{2a} ↑, IgG_{2b} ↑↑, IgM ↑↑. C.parvum + gold salts: IgG_{2a} ↑, IgG_{2b} ↑, IgM ↑.	C.parvum: ↑ C.parvum + gold salts: →	C.parvum: ↑↑ C.parvum + gold salts: ↑↑	C.parvum: ↑ C.parvum + gold salts: →
Injection of C.parvum into T-cell deprived mice	IgG_1 →, IgG_{2a} ↑, IgG_{2b} ↑, IgA ↑, IgM ↑.	Uncertain	↑	↑
Injection of different strains of C.parvum	CN 6134 : IgG_{2b} ↑ 10470 : IgM ↑, IgG_{2b} ↑. 10387 : IgM ↑, IgG_{2b} ↑, IgG_1 ↑.	CN 6134 : ↑ 10470 : ↑ 10387 : ↑↑	N.T.	↑ → ↑
Injection of different adjuvants	C.parvum : IgA ↑, IgM ↑, IgG_1 ↑, IgG_{2a} ↑↑, IgG_{2b} ↑↑. B.pertussis : IgM ↑, IgG_1 ↑, IgG_{2a} ↑, IgG_{2b} ↑. B.C.G. : IgG_1 ↑, IgG_{2b} ↑.	C.parvum: ↑ B.pertussis : →	N.T.	↑ → →

TABLE 46

POSSIBLE SEQUELAE TO C.PARVUM INJECTED SYSTEMICALLY



suggested that cancer patients (melanoma and breast cancer) had higher serum IgA and IgG than controls. However, it was unclear how many of the patients were undergoing BCG therapy at the time (194).

Results from this laboratory have consistently shown that certain immune responses, in mice with an established CBA fibrosarcoma, are not decreased and may be elevated. Thus, the IgM and IgG responses to SRBC and pneumococcal polysaccharide (Type III) were at least as great as controls (183); the IgM and IgG_{2b} responses to SRBC were elevated (184); and K-cell activity of spleen and lymph nodes was increased (175). The response to alum-BSA, however, may be reduced in mice with established tumours (184).

The effect of simultaneous injection of tumour and antigen on the response to that antigen is more variable. Thus, the response to SRBC was unaffected, the response to pneumococcal polysaccharide (Type III) was enhanced, and the response to alum-BSA was reduced (183). Further studies in this context showed that the IgG_1 response to SRBC was depressed (184) and that K-cell activity was only present in the spleens of mice with established tumours (195).

These studies were extended by examining the effect of transplanted plasmacytomas on the humoral responses to SRBC and alum-BSA (Section 7). It was apparent that simultaneous injection of tumour cells and SRBC could result in a decrease in responsiveness to SRBC, whereas injection of antigen into mice with established tumours could enhance responsiveness to SRBC. In addition the response to alum-BSA was reduced when given 5 days after tumour transplantation.

Thus, in general, simultaneous injection of tumour and antigen

more effectively depresses the immune response to the antigen than does injection of antigen into mice with an established tumour. Furthermore, K-cell activity is directly related to the size of the tumour. This being so it is difficult to see how tumour cell products might depress the immune response, as they are presumably in greater abundance in mice with established tumours. A more likely explanation is that the immune system is temporarily paralysed by the influx of a large number of tumour cells.

This would seem to be in accord with the observation by North *et al.* (196) that in mice an injection of tumour cells first resulted in a state of severely suppressed anti-bacterial resistance (and resistance to tumour challenge), and then in a contrasting state of greatly enhanced anti-bacterial resistance (and concomitant tumour immunity).

Finally, section 6 examined the effects of various immunotherapeutic regimes on tumour growth, with rather unsatisfactory results. An attempt to utilise any embryonic antigen, hypothetically present on the tumour cell surface, to inhibit tumour growth led to inconclusive results. Reasons for this were put forward in Discussion 2. Attempts to influence the hosts capacity to deal with tumour cells, by coating of tumour cells with antibody prior to transplantation, raised the distinct possibility of some form of enhancement (Table 38).

With remarkable consistency Prehn has argued that a weak immunological reaction leads to tumour enhancement, whereas a strong one leads to tumour rejection (8). Thus, the development of fewer tumour colonies in the lungs of athymic "nude" mice after i.v. injection of tumour cells would be explained by the incipient immune response

being enhancing (197). A corollary of Prehn's theory is that an immunological reaction against "weak" tumour antigens leads to enhancement, and a reaction to "strong" tumour antigens leads to rejection. Embryonic antigens would be counted as weak tumour antigens and this might explain the enhancement in this system and in a similar system described by Baldwin et al. in which i.v. administered tumour cells of low immunogenicity, treated by i.v. BCG, showed enhanced growth (198). In this context the enhancement of tumour growth with antiserum raised against embryonic cells might be considered (112).

The difference between "weak" and "strong" tumour antigens may reside partly in their density on the cell surface, as a methylcholanthrene induced tumour in $C_{57}Bl$ mice is known to exhibit considerable amounts of surface embryonic antigen, and is one of the few tumours amenable to prophylaxis with embryonic antigen (43).

Tumour antigens are not the only examples of antigens which, paradoxically, are known to exist but are of limited value to the host in combating the proliferation of cells possessing the antigen. Thus, protozoans and other pathogens can exist in an immunologically hostile environment (199), and hosts will succumb to growth of tumour cells which are H-2 identical but different at minor loci (200) (whereas they reject tumour cells that are not H-2 identical).

Surely then the problem is one of manipulation of the host and/or tumour cells with the object of promoting an effective response against weak tumour antigens. This may be envisaged as a quantitative boosting in the level rather than a qualitative shift in the type of response and, if the foregoing is true, spontaneous tumours, or tumours

of low immunogenicity, should increasingly occupy the time of investigators. Ways of augmenting the response to weak antigens were discussed in the Introduction (section 7); however, since then further data has been unearthed which supports the line taken here.

For instance, consider the B16 melanoma in $C_{57}Bl$ mice, an especially malignant tumour which readily metastasizes to the lung and contains a low percentage of macrophages when grown subcutaneously (103). The unmodified tumour cells were unable to sensitise syngeneic spleen cells *in vitro* against the tumour antigen. However, syngeneic spleen cells co-cultured *in vitro* with chemically modified B16 tumour cells were thereby rendered specifically cytotoxic (201). Despite its capacity to invade other organs this tumour promotes an immune response as measured by deposition of antigen-antibody complexes in the kidneys of mice with a progressing tumour (202).

BCG has also been used to augment responses to tumours of low immunogenicity. Thus, Hopper *et al.* (203) and Bartlett *et al.* (204) were able to suppress the growth of tumour cells by injecting them in mixed inocula together with the adjuvant. In the first instance (203) no protection to rechallenge was obtained, in the second it was (204). The reason for this may be that the "antigenic load" was not large enough in the former case. Thus, it has been shown that if tumour growth is suppressed totally by adjuvant then injection of a mixture of tumour cells and BCG is relatively ineffective in conferring protection against rechallenge. However, infiltration of a growing tumour with BCG, followed by excision of the growing tumour, is more effective than excision alone at immunising the host (205).

To conclude, it might be appropriate to consider ways in which the work described might be developed. It would appear that the antibody produced by systemic injection of C.parvum has, if anything, an enhancing effect on transplanted tumour cells. The greatest effect is generally produced using serum from C.parvum treated mice; however, there is a difference between the antibody produced by injection of C.parvum into normal mice and injection into tumour bearing mice. Fractionation of such sera and their use in Winn assays should yield information of interest.

Further to this, the Winn assay may be made more sensitive by injecting a range of tumour cell numbers and dilutions of serum. It may be significant that such assays only seem to work with a vast preponderance of effector cells to target cells.

It might also be timely to perform a similar detailed analysis on other models of tumour immunity such as contact sensitivity, produced by injecting tumour cells in contact with adjuvant or infiltrating a growing tumour with adjuvant. Such procedures are considered to influence tumour growth by specific immunological means, and therefore might be expected to emphasize the 7S response at the expense of the 19S response.

Methods to examine the class and sub-class of immunoglobulin adhering to cells of a freshly excised tumour might also be explored. In theory this should be a more reliable measure of tumour-antibody interactions than the in vitro assay used in these studies. However, the separation of Fc-receptor bearing cells from tumour cells, without dislodging any immunoglobulin, appears at first sight to be a formidable problem.

'and what is the use of a book,' thought Alice, 'without pictures or conversations?'

from "Alice's Adventures in Wonderland

by Lewis Carroll

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